DRUG-INDUCED STIMULATION OF THE GLUCURONIC ACID SYSTEM

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A STUDY ON THE DRUG-INDUCED ENHANCED METABOLISM OF CARBOHYDRATE VIA THE GLUCURONIC ACID SYSTEM IN MAMMALS

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
DEAE-cellulose	diethylaminoethylcellulose
DNA	deoxyribonucleinic acid
EC No	enzyme commission number
i.p.	intraperitoneally
i v.	intravenously
NAD	nicotinamide adenine dinucleotide
$NADH_2$	nicotinamide adenine dinucleotide in reduced form
NADP	nicotinamide adenine dinucleotide phosphate
$NADPH_2$	nicotinamide adenine dinucleotide phosphate in reduced form
P_i	inorganic phosphate
RNA	ribonucleinic acid
s c.	subcutaneously
S D.	standard deviation
UDP	uridine diphosphate
UDPG	uridine diphosphate glucose
UDPGA	uridine diphosphate glucuronic acid
UMP	uridine monophosphate

INTRODUCTION

There is growing evidence from recent publications that many drugs, besides their well-known pharmacodynamic actions, display other effects especially as far as their influence on drug-metabolizing enzymes in the liver is concerned. Moreover, their stimulating effect on carbohydrate metabolism via the glucuronic acid system should be mentioned in this respect. Drugs belonging to essentially different pharmacological groups appear to have in common the initiation of these phenomena.

Because of the possible significance of the phenomena mentioned for therapeutic and toxic effects of drugs, the biochemical backgrounds of these phenomena are being intensively studied (Conney, 1967).

In the study described in this thesis the stimulating effect of drugs on carbohydrate metabolism via the glucuronic acid pathway takes a central position.

The term 'glucuronic acid pathway' has been introduced by Burns (1959). By this he means the formation of D-glucuronic acid from D-glucose. The discovery that D-glucuronic acid can be processed further via D-xylulose and pentose phosphate resulted in an extension of the concept of the glucuronic acid pathway. We will use the wider term 'glucuronic acid system' for the sequences of biochemical processes leading from D-glucose or D-galactose to D-glucuronic acid and those leading from D-glucuronic acid to the various products such as D-glucaric acid, L-ascorbic acid and D-xylulose (Fig. 1).

In the glucuronic acid system D-glucuronic acid has a key-position as a metabolic intermediate in the biosynthesis of L-ascorbic acid, D-glucaric acid and D-xylulose. The intermediate D-glucuronic acid as well as the products mentioned, are excreted in the urine of mammals. The stimulating effect of drugs on the glucuronic acid system becomes manifest by the enhanced excretion in the urine of one or more of the compounds mentioned. Neither the pharmacodynamic properties of the drugs, nor their chemical structures appear to be of special significance for their stimulating capacity.

Greenwald (1930) observed an increased excretion of pentose in patients with congenital pentosuria during treatment with borneol, aminophenazone or phenazone. Enklewitz and Lasker (1935) identified this pentose as L-xylulose and also observed that after giving D-glucuronic acid as a lactone to patients with pentosuria an increased excretion of L-xylulose in the urine takes place.

Longenecker et al. (1940) reported that in the urine of rats treated with barbiturates or pyrazolone derivatives the quantity of L-ascorbic acid in 24-hr periods, largely surpasses the total normal content of L-ascorbic acid in the

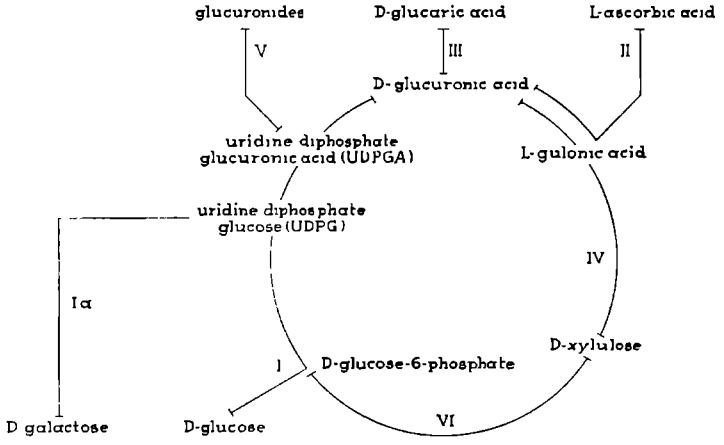

FIG. I *The glucuronic acid system and related pathways.*

I and Ia The glucuronic acid pathway. The formation of D-glucuronic acid from D-hexoses takes place along this pathway.

II The ascorbic acid pathway which results in the formation of L-ascorbic acid from D-glucuronic acid.

III The glucaric acid pathway which results in the formation of D-glucaric acid from D-glucuronic acid.

IV The xylulose pathway. The formation of D-xylulose takes place along this pathway. The pentose on its turn is metabolized further along the pentose phosphate pathway (VI).

V The glucuronide formation which may be linked to the glucuronic acid pathway.

VI The pentose phosphate pathway, which together with the glucuronic acid pathway (I) and the xylulose pathway (IV) forms the glucuronic acid cycle.

body. These investigators postulated that either an increased biosynthesis or an inhibition of the degradation of L-ascorbic acid in the organism is involved. Examples of other drugs which bring about an increased excretion of L-ascorbic acid are the muscle relaxants orphenadrine and meprobamate, the antihistaminics diphenhydramine and chlorcyclizine, the antirheumatic drug phenylbutazone (Conney and Burns, 1961), the neuroleptic drug chlordiazepoxide (Hoogland et al., 1966) and the insecticide DDT (Aarts, 1968). By means of tracer studies, using radio-active D-hexose, Burns et al. (1957) and Evans et al. (1960) were able to prove that the increased excretion of L-ascorbic acid takes place through an increased biosynthesis via D-glucuronic acid. The increased excretion in the urine of L-ascorbic acid as well as of L-xylulose could be ascribed to an increased formation of D-glucuronic acid in the liver cells

(Conney et al., 1961). An increased formation of D-glucuronic acid is also manifested in an increased excretion of D-glucuronic acid in the urine during treatment of animals (rat and guinea pig) with barbital (Burns et al., 1957).

In the past 10 years much attention has been paid to the phenomenon of enhanced enzyme activities in liver tissue, caused by drugs. One of the aspects most intensively studied is the drug-induced enhanced capacity of drug-metabolizing enzyme systems in the liver. Brown et al. (1954) reported in rats an enhanced metabolism of drugs by liver tissue after treatment with some carcinogenic polycyclic hydrocarbons. From other experiments (Conney et al., 1956, 1957a, b) it appeared that probably an increased synthesis of drug-metabolizing enzyme systems is involved. Conney and Burns (1959) and Remmer (1959) reported that also drugs such as the sedative phenobarbital, the antipyretic aminophenazone and the antirheumatic drug phenylbutazone can act as inducers of enhanced drug metabolism in the rat.

Clinically, similar observations were made. It appeared that in patients treated for some time with phenobarbital or phenylbutazone the elimination of other drugs such as the anticoagulant dicoumarol (Dayton et al., 1961), the antipyretics novaminsulfone (Remmer et al., 1961) and aminophenazone (Chen et al., 1962) was speeded up. These data support the idea that not only in animals, but also in man during and after application of drugs an induction of an increased synthesis of drug-metabolizing enzyme systems takes place. After these initial observations many other experimental examples supporting this idea were reported in the literature (Burns and Conney, 1965; Cucinell et al., 1965, 1966; van Dam, 1968). The microsomal drug-metabolizing enzyme systems in the liver, which appear to be involved in the induction phenomenon as a rule show little specificity, which means that a variety of drugs can be converted by the same enzyme system (Gillette, 1966). Moreover, the induction phenomenon also shows little specificity. One drug brings about an increased activity in various enzyme systems, and various unrelated drugs can induce an increased activity of the same enzyme system. These relationships imply that it is hardly possible to combine drugs which are really independent in this respect. This is of great consequence for pharmacotherapy. As a rule the dosis of a drug A is chosen in such a way that via an optimal concentration of it in plasma or at the site of action the wanted therapeutic effect is obtained. Application of a drug B, given before or simultaneously, will often result in an enhanced metabolism and in an attendant increase of the inactivation of drug A, resulting in an interference with its therapeutic effect. Besides this, drug A as well as drug B may act as inducers of the enzyme systems involved in their own biochemical degradation. May be that part of the unexpected and partly undetected complications after application of combinations of drugs find their origin in the phenomena

TABLE I *Drugs with a stimulating influence on drug metabolism in rat liver and on the urinary excretion of L-ascorbic acid in rats.*

Function	Drug	Stimulating influence on drug metabolism	*Ref.*	Ratio of L-ascorbic acid production in urine: during drug treatment/ before drug treatment	*Ref.*
Analeptic	Nikethamide	+	l	4	o
Analgesic	Aminophenazone	+	b	35	a
	Phenazone	+	b	30	a
Antidiabetic	Chlorpropamide			2	o
	Tolbutamide	+	n	1.1	o
Ataractic	Chlordiazepoxide	+	d	6	d
Antihistaminic	Diphenhydramine	+	c	5	c
	Chlorcyclizine	+	c	18	c
Antirheumatic	Phenylbutazone	+	b	12	b, c
	Salicylic acid			2	a
Antiseptic	Borneol			2	o
Hypnotic	Barbiturates	+	b	6–50	a, b, c
	Chloretone	+	b	40	a
	Bromisoval			10	a
	Paraldehyde			50	a
Muscle relaxant	Orphenadrine	+	b	20	a, b
	Meprobamate	+	b	7	a, b
Uricosuric	Sulfinpyrazone			7	c
Antoxidant	Butylated hydroxytoluene	+	e	>1	e
	Butylated hydroxyanisole	+	e	>1	e
Carcinogenic	3-Methylcholanthrene	+	b	30	b
	3,4-Benzpyrene	+	k	20	b
Insecticide	DDT	+	m	5	o
Inhibitor of drug metabolism	SKF 525A*	+	m	5	f, g
Inducer of porphyria	Sulphonal			27	h
	2-Allyl-2-isopropylacetamide			24	h

* This drug displays a diphasic effect: first phase a diminished capacity for drug metabolism, dependent on the presence of the drug in the liver, and second phase, an enhanced capacity for drug metabolism which may go on for a certain time after elimination of the drug.

Ref.:

a Longenecker et al., 1940
b Conney and Burns, 1959
c Burns et al., 1960
d Hoogland et al., 1966
e Gilbert and Goldberg, 1965
f Kato et al., 1962
g Neumann et al., 1963
h de Matteis, 1964
i Baumann et al., 1942
j Conney et al., 1960
k Burns et al., 1963
l Brazda and Baucum, 1961
m Gerboth and Schwabe, 1964
n Remmer, 1962
o This thesis

under discussion. In that case a better understanding of these phenomena may in the future convert these unexpected reactions into expected, so that undesirable complications can be avoided.

For many of the drugs which act as inducers of drug-metabolizing enzyme systems the effect on the L-ascorbic acid excretion, especially in the rat, has also been studied (Table 1). In all cases the drugs studied were found to be capable of evoking an increased excretion of L-ascorbic acid. As mentioned before, the increase in the excretion of L-ascorbic acid is found to be based for a number of drugs on the stimulation of carbohydrate metabolism via D-glucuronic acid. Because of the co-incidence of the latter phenomenon with the induced increase in drug metabolism, a direct relationship or biochemical link between them is assumed to exist. A strong argument in this respect is found in the observation that after treatment of animals with strong inducers of drug-metabolizing enzyme systems in the liver such as chloretone, barbital, aminophenazone or the carcinogenic hydrocarbons, the activities of some of the enzymes involved in the glucuronic acid system such as uridine diphosphate glucose dehydrogenase and uridine diphosphate glucuronyltransferase in the liver are also increased (Conney et al., 1960; Hollmann et al., 1962). Treatment of the animals with ethionine, an inhibitor of protein synthesis, suppressed the induction of the enhanced drug metabolism as well as the enhanced L-ascorbic acid excretion (Touster et al., 1960; Hollmann et al., 1962).

The stimulation of the glucuronic acid system by drugs has been studied less extensively than that of the drug-induced enhanced drug metabolism. In humans, besides the observations of the increased L-xylulose excretion by patients with pentosuria after treatment with phenazone, aminophenazone or borneol, no definite information on the stimulation of the glucuronic acid system is available.

As indicated already the effects of drugs on drug metabolism may be of great significance to therapy with regard to the therapeutic effects, as well as to the side-effects. The same holds true, although to a lesser degree, for the influence of drugs on carbohydrate metabolism via the glucuronic acid system. The fact that both phenomena occur simultaneously resulted in the assumption (Burns et al., 1963) that the increase in the excretion of L-ascorbic acid in the urine can be considered as an indication for an enhanced capacity for drug metabolism. For the biochemical background of the enhanced drug metabolism during and after drug treatment, reasonable progress has been made in the past years. This is not the case as far as the stimulation of carbohydrate metabolism via the glucuronic acid system is concerned.

In this thesis the main emphasis will be laid on the stimulation of the glucuronic acid system under the influence of drugs. The questions that arise in this respect and that require to be studied more closely, are:

1. Does the enhanced metabolism via the various pathways of the glucuronic acid system during drug treatment, as observed in animals, also occur in patients? In man (like in monkey and guinea pig) L-ascorbic acid cannot be used as an indicator for such an enhanced metabolism since in these species no biosynthesis of L-ascorbic acid takes place. In patients with pentosuria L-xylulose might be used as a criterion. This possibility, however, is as a matter of fact very restricted. For this reason it is worthwhile to investigate whether in man other products, especially endproducts, of the glucuronic acid system or of pathways linked to it can serve as an indicator for the stimulating effect of drugs on the glucuronic acid system.

2. What is the time pattern of the drug-induced response of the glucuronic acid system? The assumption that the increase in the excretion of L-ascorbic acid might serve as an indicator for an increased capacity for drug metabolism, is based on a few, mainly qualitative experiments. The study of the drug-induced enhanced drug metabolism as well as the drug-induced enhanced activity in the glucuronic acid system as a function of time is required in order to get a better insight in the possible interrelationships between these phenomena. Especially as far as the enhanced activity in the glucuronic acid system is concerned, additional data will have to be obtained.

3. What are the basic processes in the induction of the enhanced enzymatic activity by drugs? In order to get a better insight in the basic processes involved in the stimulation of the glucuronic acid system a more detailed analysis of the significance of certain hormones and of certain enzymatic steps respectively, in the glucuronic acid system for the phenomena under consideration was set as

a further goal. Moreover, a comparison should be made between the drug stimulation of the pentose phosphate pathway and that of the glucuronic acid system.

4. Is there a functional link between the glucuronidation of drugs or drug metabolites and the stimulation of the glucuronic acid system? Often metabolism of drugs implies the formation of glucuronides as a result of the transference of the glucuronyl moiety from uridine diphosphate glucuronic acid to the drugs or their metabolites. Uridine diphosphate glucuronic acid is an intermediate of the glucuronic acid system. In this light the stimulation of the glucuronic acid system by drugs may be of special significance. In the literature there are indications for an increased glucuronidation of drugs or their metabolites under the influence of barbiturates, which are stimulators of the glucuronic acid system (Büch et al., 1967).

In the following chapters a review will be presented of the literature with respect to the glucuronic acid system and with respect to the effect of drugs on it, followed by the experiments performed to get answers to the questions posed. After that the results obtained will be discussed.

CHAPTER I

THE FORMATION AND CONVERSION OF D-GLUCURONIC ACID

1.1 Introduction

The stimulation of carbohydrate metabolism via the glucuronic acid system by drugs is manifested by an increased excretion of D-glucuronic acid and also of products formed from it such as L-ascorbic acid and L-xylulose. For the study and the analysis of the various phenomena related to the glucuronic acid system, insight in the various metabolic pathways and enzymatic steps related to and involved in them is required. Of special importance are those steps and products which, although not studied in relation to the stimulation phenomenon up to now, may possibly be suitable indicators for the detection of an increased activity and suitable points of attack for the analysis of the glucuronic acid system. The formation of D-glucaric acid from D-glucuronic acid and also the formation of glucuronides can be mentioned in this respect. Thorough reviews on the metabolic systems involved in D-glucuronic acid and glucuronide formation are written by Touster and Shaw (1962) and Dutton(1966). In the past 2 years new information and new points of view have arisen, especially as far as the formation of D-glucuronic acid and its conversion to other products are concerned. This implies that a discussion of the glucuronic acid system, taking into account these new aspects, is suitable.

The various pathways, sequences of biochemical conversions which are named after the endproducts and in which D-glucuronic acid is the end- or the starting product, will be discussed in the following sections (Fig. 1):

I. The glucuronic acid pathway leading from D-glucose to D-glucuronic acid or from D-galactose to D-glucuronic acid (Ia). The possibility of the formation of D-glucuronic acid by hydrolysis of polysaccharides containing D-glucuronic acid will also be discussed briefly.

II. The ascorbic acid pathway leading from D-glucuronic acid to L-ascorbic acid. For certain mammals, namely man, monkey and guinea pig, L-ascorbic acid is a vitamin since one of the enzymes essential in the L-ascorbic acid pathway is lacking. This implies that in the discussion of this pathway mainly other mammals will be involved.

III. The glucaric acid pathway leading from D-glucuronic acid to D-glucaric acid. This short pathway consists of only two steps with D-glucuronolactone as an intermediate. The pathway was identified by Marsh (1963a).

IV. The xylulose pathway, leading from D-glucuronic acid to D-xylulose. In certain patients the step in this pathway leading from L-xylulose to xylitol is absent because of an inborn error in metabolism, based on an enzyme defect. The consequence is an overflow of L-xylulose which appears in the urine of the patient (essential pentosuria).

The xylulose pathway is linked via D-xylulose-5-phosphate with the pentose phosphate pathway (VI). The pentose phosphate pathway leads to D-hexose phosphate, which in its turn can be taken up in the glucuronic acid system. Herewith a cycle of biochemical conversions is closed, implying that the various pathways mentioned can be considered as a part or as side-ways of what is called in the literature the glucuronic acid cycle.

V. The glucuronide formation. This conjugation reaction may be linked to the glucuronic acid system. The glucuronyl moiety is transferred from uridine diphosphate glucuronic acid (UDPGA) to the acceptor (drugs, drug metabolites and body-formed compounds such as bilirubin and thyroxin). The conjugation reaction possibly constitutes one of the main links between the glucuronic acid system and drug metabolism.

1.1.1 The formation of D-glucuronic acid

It is assumed that D-glucuronic acid is mainly formed by biosynthesis via UDPGA (glucuronic acid pathway). In addition the possibility exists that D-glucuronic acid is formed by catabolism of polysaccharides containing this compound.

1.1.1.1 The glucuronic acid pathway

Although the occurrence of D-glucuronic acid as a physiological substance was revealed in 1879 (Schmiedeberg and Meyer), an insight in the nature of the intermediate steps of the biosynthesis of this substance has been gained only during the last decade. The isolation of UDPGA was of essential importance in this respect (Dutton and Storey, 1954; Smith and Mills, 1954). The enzymes and products involved in this biosynthesis are presented in Fig. 2.

Uridine diphosphate glucose dehydrogenase

One of the steps linking intermediates of glycolysis with D-glucuronic acid metabolism was established with the discovery (Strominger et al., 1957) that the liver contains an enzyme capable of oxidizing uridine diphosphate glucose (UDPG) to UDPGA in the presence of nicotinamide adenine dinucleotide (NAD). This soluble enzyme, the UDPG dehydrogenase, acts on its substrate

in two clearly separated steps. In the first step only the uracil moiety of UDPG appears to be oxidized at the expense of NAD to produce an as yet uncharacterized substance (XUDPG). In the second step this substance is further oxidized with NAD and in the course of this reaction the oxidized uracil moiety is restored to its original configuration, while the carbon atom 6 of the D-glucose moiety is oxidized to a carboxyl group (Simonart et al., 1966). It seems that

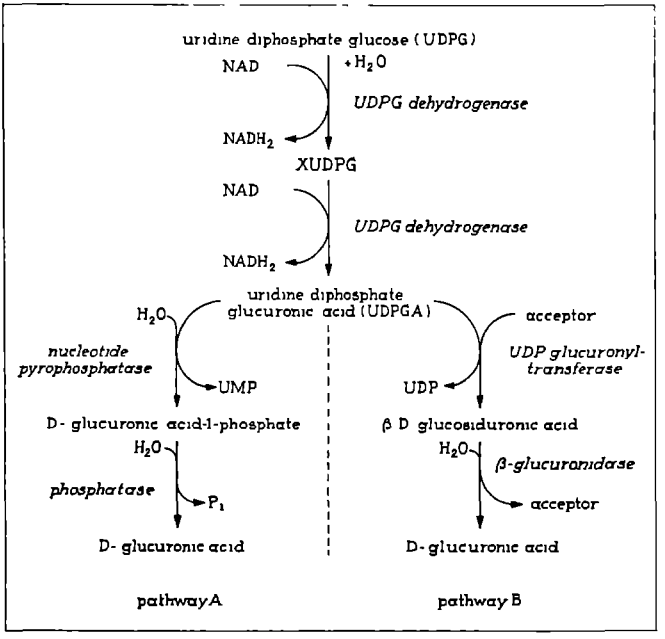

FIG. 2 *The glucuronic acid pathway.*

Note It is not established whether D-glucuronic acid formation *in vivo* takes place via pathway A or via pathway B.

these reactions are not reversible. The UDPG dehydrogenase is specific for the substrate UDPG; D-glucose and D-glucose-1-phosphate cannot be used as substrates by the enzyme. The pH optimum of the purified enzyme is 8.7.

It has been demonstrated that the UDPG dehydrogenase isolated from bovine liver is inhibited competitively by UDPGA (K_I 5 x 10^{-5}M), by uridine diphosphate xylose (K_I 4 x 10^{-8}M) (Neufeld and Hall, 1965) and by uridine diphosphate galactose. Moreover, an uncompetitive inhibition by uridine diphosphate (UDP) has been reported (Salitis and Oliver, 1964). The question whether these inhibiting compounds exercise a regulatory function in the glucuronic acid pathway awaits further experiments.

The formation of D-glucuronic acid from UDPGA in the liver has been demonstrated clearly with tracer studies (Evans et al., 1960; Pogell and Leloir, 1961; Conney and Burns, 1961). *In vitro* two possible pathways (A and B, Fig. 2) have been demonstrated; whether these pathways play a role also *in vivo* is not sure. The intermediate enzymatic steps involved *in vivo* have not yet been established with certainty.

Nucleotide pyrophosphatase and phosphatase

The pathway A is catalyzed by a nucleotide pyrophosphatase and a phosphatase. The first enzyme catalyzes the conversion of UDPGA into D-glucuronic acid-1-phosphate and uridine monophosphate (UMP). The phosphatase converts D-glucuronic acid-1-phosphate into D-glucuronic acid and inorganic phosphate (P_i). Using rat kidney microsomes and starting from UDPGA as a substrate Ginsburg et al. (1958) were the first to show the formation of D-glucuronic acid-1-phosphate and D-glucuronic acid. The formation of D-glucuronic acid-1-phosphate and D-glucuronic acid from UDPGA could also be demonstrated with the aid of rat liver microsomes (Pogell and Leloir, 1961; Conney and Burns, 1959, 1961).

Recently, the purification of pyrophosphatase from the rat liver was performed. The pyrophosphatase is present in the microsomal fraction and in the nuclei; the supernatant shows little pyrophosphatase activity. Schliselfeld et al. (1965) and Ogawa et al. (1966) studied purified pyrophosphatase obtained from the nuclei and from the microsomal fraction respectively. In both cases the purified enzyme had its highest activity at alkaline pH (the microsomal enzyme at pH 9.1; the enzyme obtained from the nuclei at pH 8.5). Little substrate specificity was observed; for instance, the substrates UDPG, UDP-N-acetylglucosamine and NAD were also hydrolyzed. It is remarkable that the microsomal fraction from guinea pig liver shows little or no pyrophosphatase activity (Pogell and Leloir, 1961).

The phosphatase activity of the rat liver, tested with D-glucuronic acid-1-phosphate as a substrate is very low, this in contrast with the phosphatase activity obtained from the rat kidney (Pogell and Leloir, 1961; Conney and Burns, 1961; Takanashi et al., 1966). The kidney phosphatase which hydrolyzes D-glucuronic acid-1-phosphate is mainly present in the microsomal fraction. This enzyme was purified by Takanashi et al. (1966); according to these authors it is identical with a nonspecific alkaline phosphatase (EC 3.1.3.1). Whether the liver phosphatase, which hydrolyzes D-glucuronic acid-1-phosphate, is involved in the glucuronic acid pathway requires further investigation. In view of the low activity of this enzyme it is not clear whether the pyrophosphatase and phosphatase are involved in the D-glucuronic acid formation.

UDP glucuronyltransferase and β-glucuronidase

As pointed out by Pogell and Leloir (1961) the combination of microsomal UDP glucuronyltransferase and microsomal β-glucuronidase may be involved in the formation of D-glucuronic acid by the liver.

UDP glucuronyltransferase catalyzes the transfer of the glucuronyl moiety from UDPGA to the acceptor (Fig. 2). The properties of the glucuronyl transferase and the glucuronide formation will be discussed further in Section 1.1.5.

The enzyme β-glucuronidase hydrolyzes the glucuronide under the formation of the free D-glucuronic acid and the acceptor. The enzyme has a low specificity. It is strongly inhibited by D-glucaro-(1→4)-lactone and to a lesser degree by D-glucuronic acid (product inhibition; Levvy, 1952). Whether these inhibitors are of physiological significance is not clear (Marsh, 1964; Fishman, 1964).

In the study of Pogell and Leloir (1961) on the influence of UDP-N-acetylglucosamine on the enzymatic formation of D-glucuronic acid, D-glucuronic acid-1-phosphate and glucuronide by rat liver microsomes, a strong indication is found that both UDP glucuronyltransferase and β-glucuronidase are of real importance for the D-glucuronic acid formation, at least *in vitro*. From this study it follows that UDP-N-acetylglucosamine is an activator of UDP glucuronyltransferase *in vitro*. It appears that this compound enhances the formation of D-glucuronic acid from UDPGA and simultaneously decreases the formation of D-glucuronic acid-1-phosphate from the same substrate. The possible explanation might be the following: the pathway A (Fig. 2) is inhibited because UDP-N-acetylglucosamine competes with UDPGA for the pyrophosphatase whereas simultaneously the pathway B is stimulated by the activated UDP glucuronyltransferase.

If the combination of UDP glucuronyltransferase and β-glucuronidase plays a role in the physiological D-glucuronic acid formation, the presence of an endogenous acceptor for the glucuronyl moiety is required. Up till now such an acceptor could not be detected. It follows that a function of UDP glucuronyltransferase and β-glucuronidase in the D-glucuronic acid formation *in vivo* is not established (see also Miettinen and Leskinen, 1963).

1.1.1.2 The formation of D-glucuronic acid by catabolism of polysaccharides

D-glucuronic acid is found copolymerized in 1:1 ratio with substituted 2-amino-2-deoxy-D-glucose in compounds as heparin, chondroitin sulfate-A and -C, and hyaluronic acid (Whistler and Rowell, 1966). Besides D-glucuronic acid biosynthesis, catabolism of such compounds may be a source for D-glucuronic acid in the cell.

Enzymes for the breakdown of D-glucuronic acid containing polysaccharides

are present in the liver. It was found by Hutterer(1965) that enzymes of liver lysosomes show hyaluronidase activity. This investigator observed that the system used degrades hyaluronic acid, chondroitin sulfate-A and -C into oligosaccharides. Aronson and Davidson(1965) have isolated and partially purified hyaluronidase from rat liver lysosomes. After incubation of hyaluronidase with hyaluronic acid, oligosaccharides were formed which appeared to be identical to those resulting from degradation of hyaluronic acid by testicular hyaluronidase. β-Glucuronidase and β-acetylaminodeoxyglucosidase act alternately on the oligosaccharides (Weissmann et al., 1964). β-Acetylaminodeoxyglucosidase in rat liver is only found in the lysosomes (Sellinger et al., 1960); β-glucuronidase can be of microsomal and lysosomal origin (de Duve et al., 1955). As may be clear from the foregoing, the combined actions of lysosomal enzymes may lead to the liberation of D-glucuronic acid from polysaccharides. It is not sure whether this route of D-glucuronic acid formation has a physiological significance. Recent results suggest that lysosomes are able to engulf hyaluronidase substrates, which are then removed probably by degradation (Aronson and Davidson, 1967).

It was postulated by Laborit (1964) that D-glucuronic acid-containing polysaccharides present in the cell, may be degraded and that the remaining D-glucuronic acid is metabolized further via the xylulose pathway.

1.1.2 The ascorbic acid pathway

Experiments with labeled D-glucuronolactone and L-gulonolactone applied to rats revealed that an important proportion of these substances is converted to L-ascorbic acid (Horowitz and King, 1953; Burns and Evans, 1956). These findings were supported by the results of Isherwood et al. (1954), who have demonstrated that formation of L-ascorbic acid in intact rats is increased after administration of D-glucuronolactone and L-gulonolactone. These observations, together with other considerations, led to the postulate that D-glucuronolactone and L-gulonolactone are intermediates in the pathway leading from D-glucose to L-ascorbic acid.

The data mentioned above could be used in the identification of the enzymes concerned in the biosynthesis of L-ascorbic acid (Fig. 3).

Glucuronolactone reductase

The reduction of D-glucuronic acid to L-gulonic acid and of D-glucurono-(6→3)-lactone (D-glucuronolactone) to L-gulono-(1→4)-lactone (L-gulonolactone) respectively is catalyzed by a nicotinamide adenine dinucleotide phosphate (NADP)-linked glucuronolactone reductase. The enzyme is found in

different organs of various species. Its occurrence is not related to the ability of these tissues to synthesize L-ascorbic acid. The presence of the enzyme has been shown for the first time by Hassan and Lehninger (1956) in liver extract. Mano et al. (1961) purified the liver enzyme. They found that the enzyme is not bound to the microsomes and has its optimal activity at pH 9.6 to 10.0. The enzyme catalyzes both reduction as well as oxidation reactions. For the reduction reaction with aldehydes as hydrogen acceptors it shows little specificity; as hydrogen donors on the other hand the enzyme accepts only L-hexonic acids or their lactones.

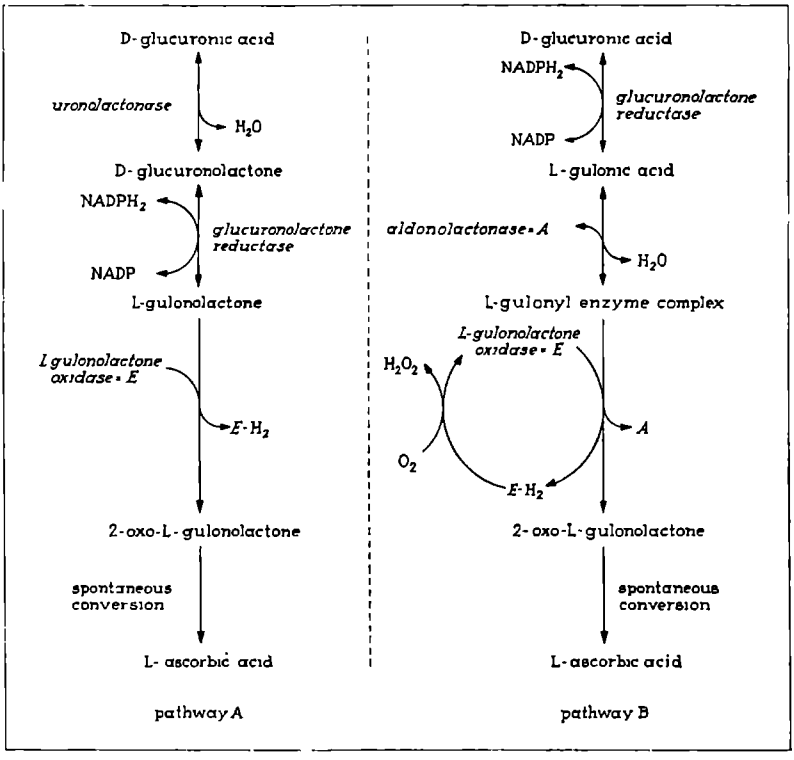

FIG. 3 *The ascorbic acid pathway.*

Note It is not established whether L-ascorbic acid formation *in vivo* takes place via pathway A or via pathway B.

L-gulonolactone oxidase

The L-gulonolactone oxidase is involved in the enzymatic conversion of L-gulonolactone to 2-oxy-L-gulonolactone, which is followed by a spontaneous

enolization to L-ascorbic acid. The description of the enzyme was given by Chatterjee et al.(1960) and Isherwood et al.(1960). Recently Brush and May (1966) published a kinetic study on the mechanism of action of this enzyme. From their data it becomes probable that the mechanism involved is a so-called 'ping-pong' mechanism. The characteristic distinguishing this mechanism for enzyme-catalyzed oxidation-reduction reactions from other ones is that the enzyme itself is reduced by the first substrate prior to reacting with and reducing the second substrate, instead of catalyzing a direct oxidation-reduction reaction between the two substrates (Fig. 3). Using a solubilized enzyme from rat liver microsomes, Kanfer et al.(1959) and Ashwell et al.(1961) showed that only those compounds that have the hydroxyl group on C-2 in the levo configuration (as in L-gulono-, L-galactono-, and L-mannonolactone) are oxidized to an analogue of L-ascorbic acid. It is essential that the substrate is in the ester or lactone form (Isherwood and Mapson, 1961). The optimal pH for oxidase activity is between 6.5 and 7.0.

Lactonases

For the biotransformation of D-glucuronic acid to L-ascorbic acid the presence of a lactonase is needed (Chatterjee et al., 1959). In the liver of all mammals studied urono- and aldonolactonase were found to be present. These enzymes catalyze the equilibrium between the lactone and acid form. Whether a net conversion of acid to lactone takes place or the reaction goes in the opposite direction depends on the circumstances; in the absence of a 'trapping agent' for the lactone such as, for instance, hydroxylamine, the enzymatic equilibrium is in favour of the acid form.

The uronolactonase is present in the microsomal fraction of the liver cells (Winkelmann and Lehninger, 1958; Yamada et al., 1959). The enzyme has a high specificity. It accepts D-glucuronolactone as a substrate but L-gulonolactone and D-gulonolactone are not converted (Mano et al., 1961). Possibly, the formation of D-glucuronolactone as catalyzed by this enzyme is a step in the biosynthesis of L-ascorbic acid. In that case the synthesis might follow pathway A (Fig. 3).

The soluble enzyme aldonolactonase (Yamada, 1959) is capable of catalyzing the interconversion between L-gulonic acid and its lactone. The enzyme has a low specificity. Some lactones such as D-glucuronolactone and D-gulonolactone are also accepted as substrates. It is not sure whether aldonolactonase plays a role in the biosynthesis of L-ascorbic acid. The enzyme accepts L-gulonic acid, which implies that the L-ascorbic acid biosynthesis might follow the pathway via D-glucuronic acid and L-gulonic acid. It is not clear whether in

that case L-gulonolactone formation would be an obligatory step or whether only the formation of a L-gulonyl-aldonolactonase complex is needed. Stirpe and Comporti(1965) studied the L-ascorbic acid formation using a 10.000 *g* supernatant of rat liver as a catalyzing system and D-glucuronolactone as a substrate. The supernatant was supplied with NAD and adenosine triphosphate (ATP). The lactonase activity in the incubate is found to be so high that the substrate was hydrolyzed already before the L-ascorbic acid formation was appreciably started. L-gulonolactone is also quickly hydrolyzed under these circumstances. A tentative explanation which fits to these experiments has been given by Isherwood et al.(1960). They suggest that a L-gulonyl-enzyme complex formed transitorily from the free acid and the aldonolactonase is oxidized by the L-gulonolactone oxidase, possibly with transference of the L-gulonyl moiety (Fig. 3, pathway B).

1.1.2.1 Why are certain mammals unable to synthesize L-ascorbic acid?

The formation of L-ascorbic acid in mammals takes place in the liver (Chatterjee et al., 1961). Man, monkey and guinea pig are not able to synthesize L-ascorbic acid and are dependent on an exogenous supply of this substance which for them therefore is a vitamine. Burns and Evans (1956) studied the role of L-gulonolactone as a precursor of L-ascorbic acid synthesis in rats and guinea pigs. In contrast with the appreciable conversion of labeled L-gulonolactone to L-ascorbic acid in rats no detectable conversion was observed in guinea pigs. Further it was found that cellular fractions of guinea pig liver are unable to convert L-gulonolactone to L-ascorbic acid. This indicates that this conversion may be postulated as the site for the missing link in the synthesis of L-ascorbic acid in this species. Chatterjee et al.(1961) performed a comparative study on the presence in mammalian liver of the enzymes of the ascorbic acid pathway. They found that those species which are unable to synthesize L-ascorbic acid (man, monkey and guinea pig) possess all the enzymes needed for the conversion of D-glucuronic acid to L-gulonolactone but that the enzyme L-gulonolactone oxidase is absent. Baker et al.(1960, 1962) reported an increased L-ascorbic acid excretion after giving humans D-glucuronolactone to eat, but these results could not be confirmed by Chatterjee et al.(1961).

1.1.3 The glucaric acid pathway

The formation of D-glucaric acid represents another pathway of D-glucuronic acid metabolism (Marsh, 1964). In the biosynthesis of D-glucaric acid from D-glucuronic acid two enzymatic steps are involved. The first one consists in

the lactonization of D-glucuronic acid by urono- or aldonolactonase (see Section 1.1.2); the second one consists in the oxidation of D-glucuronolactone to D-glucaric acid.

Inhibitors of β-glucuronidase are known to be present in mammalian urine. Marsh (1963a) demonstrated that a substance is present in boiled urine which, although non-inhibiting under alkaline conditions, acts as a β-glucuronidase inhibitor after acidic treatment. The fact that the inhibiting action is abolished again by the subsequent alkaline treatment suggests that the inhibitor is a lactone. It was known already that acidic treatment of D-glucaric acid leads to the formation of a lactone, which has a strong inhibiting effect on β-glucuronidase (Levvy, 1952). As mentioned before, this type of inhibition is observed in human urine and in urine of other mammalian species (Marsh, 1963a). Using the inhibiting action as a test it was possible to isolate D-glucaric acid bisphenylhydrazide in a crystalline and pure form from human urine (Marsh, 1963a; Aarts, 1965; see also Section 4.3.1).

An indication for the source of urinary D-glucaric acid is given by the fact that the acid-potentiated inhibition, an indicator of the D-glucaric acid content, in mammalian urine increases considerably after oral ingestion of D-glucuronolactone. Under normal conditions an inhibition equivalent to about 10 mg D-glucaric acid is detected in 24-hr urine of humans. After giving a dose of 5 g of D-glucuronolactone this response increases to the hundredfold. On the basis of these data the enzyme system for the formation of D-glucaric acid was identified (Fig. 4).

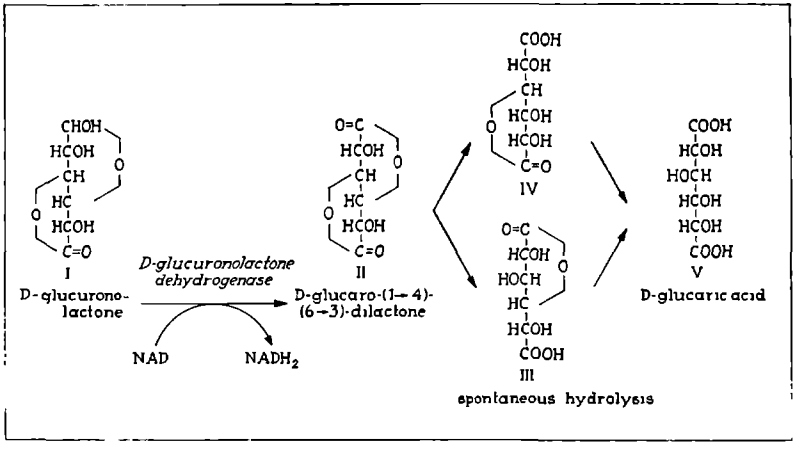

FIG. 4 *The reaction mechanism for the formation of D-glucaric acid, as proposed by Marsh (1963b).*

D-glucuronolactone dehydrogenase

This NAD-linked enzyme converts D-glucuronolactone (I) (Fig. 4) to a derivative of D-glucaric acid. Results of Marsh(1966) are consistent with the hypothesis that the (1→4), (6→3)-dilactone (II) is formed. This substance decomposes spontaneously to the (1→4)- and the (6→3)-monolactone (III, IV) and the free acid (V). Results of Sadahiro et al.(1966) are in agreement with the hypothesis that the enzyme acts upon the free aldehyde of the (6→3)-lactone of D-glucuronic acid. The enzyme in question appears to be distinct from glucuronolactone reductase, L-gulonolactone oxidase, glucose dehydrogenase and glucose-6-phosphate dehydrogenase. It does not accept D-glucuronic acid as a substrate (Marsh, 1964). The enzyme is present in the liver of all mammals examined (rat, mouse, guinea pig and man). In the rat the enzyme is found in preparations of liver, kidney and testis; in preparations of spleen, uterus, thyroid gland, lung, duodenum and in serum no enzyme activity is detectable. The enzyme activity in liver preparations is high in all species examined; the enzyme activity in kidney preparations is high in the rat, appreciable in the guinea pig and very low in mouse and man (Marsh, 1963b).

Purified D-glucuronolactone dehydrogenase from rat liver has an activity optimum at a pH of about 6.5. If the enzyme is incubated with D-glucaric acid or its lactones and $NADH_2$, no D-glucuronolactone is formed, so that no reversal of the reaction can be demonstrated.

Matsui et al.(1965) observed that after administration of D-mannuronolactone to humans a quantity of D-mannaric acid, equivalent to about 20 percent of the dose of the lactone, is found in the urine. They made the suggestion that the conversion of D-mannuronolactone to D-mannaric acid might be catalyzed by D-glucuronolactone dehydrogenase. Sadahiro et al.(1966) purified D-glucuronolactone dehydrogenase from guinea pig liver. The final purification was performed by passing the enzyme extract through a diethylaminoethyl(DEAE)-cellulose column. It was found for the fractions obtained from chromatography that the ratio of the conversion rates of D-glucuronolactone and D-mannuronolactone remains constant during the purification steps. Further no difference in heat stability was observed for the two conversions and a competitive substrate inhibition was found if both substrates are combined. These results suggest that a single enzyme is responsible for the dehydrogenation of D-glucuronolactone and D-mannuronolactone.

Sadahiro and coworkers(1966) also found an indication that D-ribose and D-ribose-5-phosphate are substrates for the dehydrogenase in question. Moreover, the experimental results obtained in DEAE-cellulose chromatography showed that the protein peak of D-glucuronolactone dehydrogenase can not be distinguished from that of aldehyde dehydrogenase (EC 1.2.1.3). It is known

that the latter enzyme is not very specific (Racker, 1949). In view of the foregoing the question arises whether the enzyme D-glucuronolactone dehydrogenase is possibly identical with this aldehyde dehydrogenase.

It is not known whether the glucaric acid pathway has a physiological significance. The pathway might be of significance if it results in the formation of the (1→4)-lactone of D-glucaric acid, which is known to be a specific inhibitor of β-glucuronidase (see before). So far the presence of this lactone in biological fluids could not be demonstrated. This does not necessarily argue against its formation, since under physiological conditions the lactone would be hydrolyzed rapidly (Levvy, 1952).

1.1.4 The xylulose pathway

This pathway, which is part of the glucuronic acid cycle, leads from D-glucuronic acid to D-xylulose (Fig. 5). It has been shown by Enklewitz and Lasker(1935) that the administration of D-glucuronolactone to patients with pentosuria causes a considerably enhanced level of L-xylulose in the urine. This suggests that this pentulose is derived from D-glucuronolactone. Touster et al. (1955) obtained similar evidence in healthy persons. In further studies with labeled compounds it could be demonstrated that the carbon atom 6 of D-glucuronolactone is lost in the conversion, whereas the carbon atom 1 of the lactone becomes carbon atom 5 in L-xylulose (Touster et al., 1957).

Subsequently it was found that L-xylulose can be formed from D-glucuronic acid as well as from L-gulonic acid, but that fewer steps are involved in the conversion starting from L-gulonic acid.

L-gulonate dehydrogenase and keto-L-gulonate decarboxylase

As mentioned before the formation of L-gulonic acid from D-glucuronic acid is catalyzed by glucuronolactone reductase (Section 1.1.2). The conversion of L-gulonic acid into L-xylulose (Fig. 5) is catalyzed by NAD-linked L-gulonate dehydrogenase and keto-L-gulonate decarboxylase (Ashwell et al., 1959; Smiley and Ashwell, 1961). The localization of this L-xylulose-forming system in rat liver is still under study. Ishikawa and Noguchi(1957) and Chatterjee et al. (1960) reported the conversion of L-gulonic acid to L-xylulose in the soluble fraction prepared from this organ. On the other hand experiments of Stirpe and Comporti(1965) indicate that the presence of the microsomal fraction is needed for the formation of L-xylulose.

L-xylulose reductase and D-xylulose reductase

Two enzymes were found to be involved in the conversion of L-xylulose to D-xylulose. They are both present in the mitochondria (Hollmann and Touster,

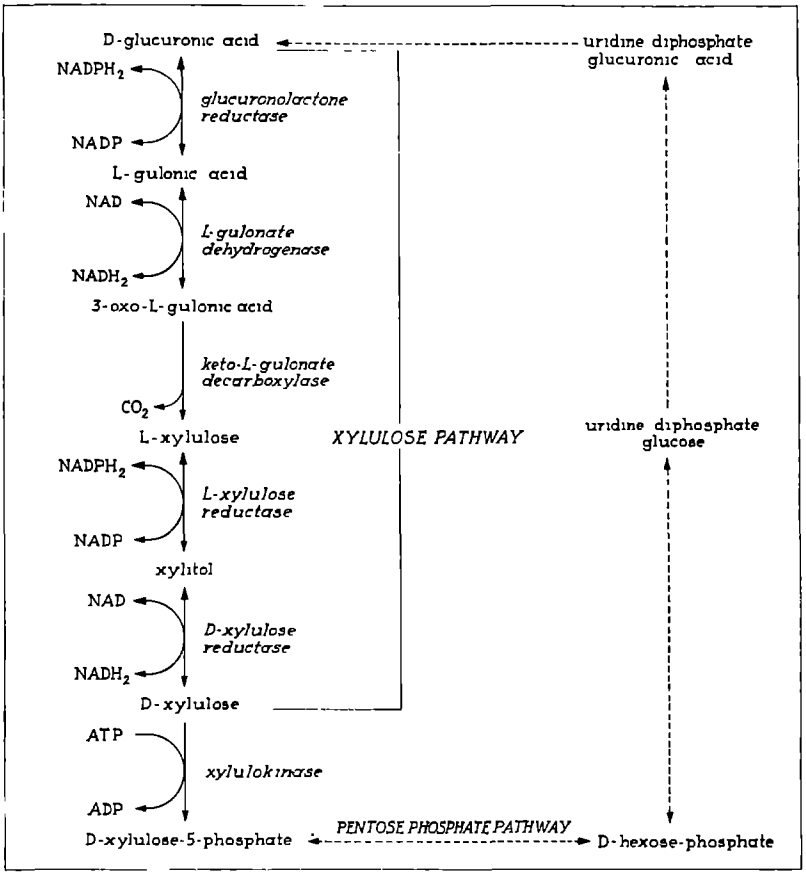

FIG. 5 *The xylulose pathway.*

Note The xylulose pathway, the glucuronic acid pathway and the pentose phosphate pathway form a cycle which is called the glucuronic acid cycle.

1956, 1957). They have been identified as a NADP-linked L-xylulose reductase of high substrate specificity catalyzing the interconversion between L-xylulose and xylitol and as a NAD-linked D-xylulose reductase with little substrate specificity. The latter enzyme may convert xylitol to L-xylulose as well as to D-xylulose; the latter product, however, is mainly formed.

The pentose phosphate pathway in the glucuronic acid cycle

The fact that an interconversion of L-xylulose and D-xylulose via xylitol was found to exist, raised the possibility that D-glucuronic acid metabolism via the xylulose pathway is linked to the pentose phosphate pathway, thus closing a

cycle. The link requires a phosphokinase, catalyzing the phosphorylation of D-xylulose to D-xylulose-5-phosphate which is a key intermediate in the pentose phosphate pathway.

Xylulokinase

Indeed the enzyme xylulokinase was reported to be present in calf liver (Hickmann and Ashwell, 1958). Thus the experimental base was laid for the closure of the glucuronic acid cycle, a cyclic pathway of D-glucose metabolism via D-glucuronic acid, L-xylulose and the pentose phosphate pathway (Burns and Kanfer, 1957) (Fig. 5).

Further evidence for the physiological occurrence of the glucuronic acid cycle was needed. It was found that L-xylulose incubated with liver slices in an appropriate medium disappears while xylitol is formed (Touster et al., 1954, 1956). In view of this finding McCormick and Touster (1957) administered labeled xylitol to rats and guinea pigs. Their experiments showed that xylitol is an efficient precursor of liver glycogen in rat and guinea pig. Administration of xylitol-1-C^{14}-5-C^{13} to rats led to a labeling pattern in glycogen which is in accord with metabolism via the xylulose pathway and the pentose phosphate pathway (McCormick and Touster, 1957; Eisenberg et al., 1959).

Essential pentosuria

Essential pentosuria is an inborn error in metabolism found in Jewish and Arab people (Hiatt, 1960). The condition is characterized by a high level of L-xylulose in the urine. The evidence that an individual with this metabolic error lacks the L-xylulose reductase is convincing (Touster, 1959) although an enzyme assay in the liver of patients with pentosuria has not been performed.

The role of the xylulose pathway is not fully elucidated. There is indirect evidence that little carbohydrate is actually metabolized through this pathway (Hiatt and Lareau, 1958). The failure to convert L-xylulose to xylitol in individuals with pentosuria is quite harmless. So the pathway is not essential for the organism. It is conceivable that the large number of oxidation-reduction reactions in the pathway helps to regulate tissue coenzyme levels (Touster and Shaw, 1962). In this connection it is remarkable that in the xylulose pathway NAD is always used in the dehydrogenations whereas $NADPH_2$ is always used in the reductive steps. Laborit (1964) postulated that the xylulose pathway may be involved in the disposal of D-glucuronic acid formed by the degradation of the polysaccharides belonging to the cell. In the experimental animals a high proportion of exogenous D-glucuronolactone is rapidly catabolyzed to CO_2 and H_2O, part of it is converted to D-hexoses and then incorporated into glycogen (Douglas and King, 1953; Butler and Packham, 1955; Burns and Evans, 1956; Eisenberg

et al., 1959). Since the xylulose pathway known so far is the only route for catabolism of D-glucuronolactone or for conversion to D-hexoses and thus to glycogen, this fact suggests that this pathway serves the elimination of surplus of D-glucuronic acid in the cell, irrespective of its source.

1.1.5 Glucuronide formation

In the following section only those aspects of the glucuronide formation, which have a special bearing on our study will be discussed in greater detail. For a more representative introduction into this subject the reader may be referred to several review articles published by Dutton (1962, 1966) and Hollmann (1964).

Many drugs and drug metabolites are eliminated from the body in the form of glucuronides. In order to be conjugated these compounds should have a reactive group, e.g. a hydroxyl, amino, sulfhydryl or carboxyl group. Drugs containing such a group function as acceptors. Glucuronide formation occurs by transfer of the glucuronyl moiety from UDPGA to the acceptor (Fig. 6).

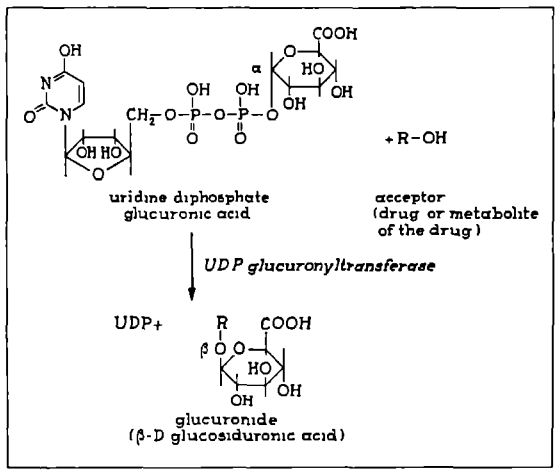

FIG. 6 *The glucuronide formation.*

Note The glucuronyl moiety in uridine diphosphate glucuronic acid occurs in α-position, but in the glucuronide in β-position.

The enzymes responsible for the transference are called UDP glucuronyltransferases (Dutton and Storey, 1954). The conjugation involves a Walden inversion, since the conjugated compounds are β-D-glucosiduronic acids, where-

as the glucuronyl moiety in UDPGA has the α-configuration. The transferase reaction is not reversible.

Glucuronidation takes place especially in the liver. Originally it was supposed that the microsomal UDP glucuronyltransferase activity in the liver is dependent on one enzyme with a low specificity and a wide pH optimum. This enzyme was thought to conjugate endogenous substrates such as bilirubin, thyroxin and steroid hormones as well as drugs and drug metabolites, substrates which normally are not present in the organism. More recent findings give a strong evidence that in the liver more than one transferase is present (Isselbacher et al., 1962; van Leusden, 1963; Dutton, 1966b; Tomlinson and Yaffe, 1966; Gram et al., 1968). Some of these findings come from developmental studies using liver tissues of fetal and very young animals. In these experiments a distinct increase of the transferase activity during development is found to occur. Measured with different phenols as substrates, quite different developmental rates of transferase activity are found (Tenhunen, 1965; Dutton, 1966b). The same holds with respect to phenols compared with bilirubin (Tomlinson and Yaffe, 1966). An obvious explanation of these results is found in the supposition that several UDP glucuronyltransferases exist.

Definite proof of the presence of more than one transferase is complicated by the fact that the transferase(s) in the liver cell is (are) connected with the endoplasmic reticulum. In this connection it may be remarked that in another tissue, viz. the ileal mucosa of man, the existence of two different transferases could be readily demonstrated. In these cells a soluble transferase catalyzing only the conjugation with aliphatic hydroxyl groups occurs. This enzyme is different from a microsomal transferase in the mucosa which catalyzes conjugations with aromatic hydroxyl groups as well as with aliphatic hydroxyl groups (Dahm et al., 1966).

If it is true that also in the liver different UDP glucuronyltransferases occur, it is conceivable that these different enzymes will serve different purposes. A function with respect to the elimination of potentially harmful substances is obvious. According to certain data (Section 1.1.1.1; Miettinen and Leskinen, 1963) a second function for UDP glucuronyltransferase is possible, viz. in the formation of D-glucuronic acid. It has been postulated that D-glucuronic acid is formed from UDPGA in the following way: first the glucuronyl moiety of UDPGA is transferred to an endogenous acceptor under the influence of UDP glucuronyltransferase, and subsequently the formed glucuronide is hydrolyzed by β-glucuronidase to give free D-glucuronic acid.

Since the glucuronidation as a way of detoxification of potentially harmful substances utilizes UDPGA, a substance taking a central position in the glucuronic acid system (Section 1.1.1.1), it may be linked to this system. The

question arises whether the UDPGA used in the various pathways of this system and that used for glucuronidation of substances to be eliminated comes from a common UDPGA pool in the liver or whether the UDPGA used for the glucuronidation and that used in the glucuronic acid system are formed in separated biochemical systems, although according to the same biochemical principles.

CHAPTER 2

STIMULATING EFFECT OF DRUGS ON CARBOHYDRATE METABOLISM VIA THE GLUCURONIC ACID SYSTEM REVIEW OF THE LITERATURE

2.1 Introduction

In this chapter investigations reported in the literature on the stimulation of carbohydrate metabolism via the glucuronic acid system caused by drugs, will be summarized and discussed. Brief reviews on this topic were written by Burns and Shore(1961) and Touster and Shaw(1962).

2.2 Characterization of the stimulation

With the term stimulation of the glucuronic acid system (see Figs. 1 and 7) is meant the stimulation by drugs or chemicals in general of metabolism in the liver of glycolytic intermediates via the pathways of the glucuronic acid system as outlined in Section 1.1. The experimental data in the literature are in agreement with the postulate that this stimulation is initiated in the glucuronic acid path-

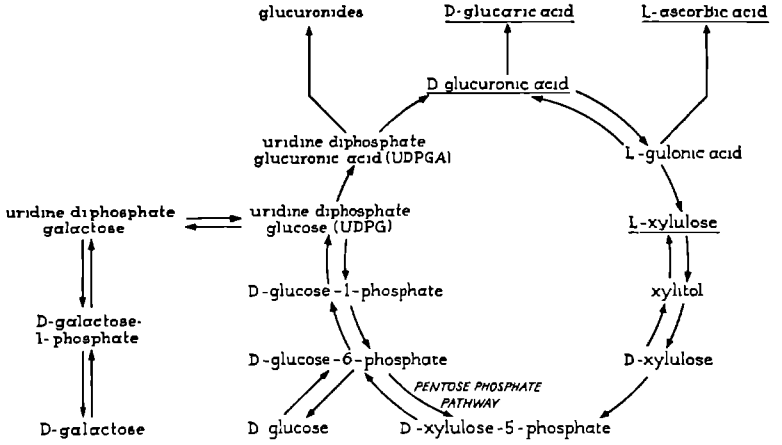

FIG. 7 *D-hexose metabolism via uridine diphosphate glucuronic acid (UDPGA).*

Note In the urine of drug-treated animals enlarged amounts of the underlined products have been detected.

way leading from D-hexose to D-glucuronic acid (Conney et al., 1961). The stimulation results in an enhanced excretion in the urine of endproducts from the various pathways of the glucuronic acid system.

The observations of the drug-induced increased excretion of products from the glucuronic acid system such as L-xylulose and L-ascorbic acid by man and rat respectively, are of much older date (Enklewitz and Lasker, 1935; Longenecker, 1940) than the identification of the enzyme systems involved in the formation of these products (Chapter 1). The increased excretion of D-glucuronic acid by rats treated with barbital as a stimulating drug is a more recent observation (Burns et al., 1957).

As will be shown in the discussion of our own results (Chapters 4 and 5) the stimulation of the glucuronic acid pathway by drugs also results in an increased excretion of D-glucaric acid by man and other mammals.

There are indications that stimulation of the glucuronic acid system is not confined to mammals. It has been shown recently that treatment of fishes with aminophenazone or barbital results in an enhanced level of L-ascorbic acid in the liver and that the hepatic enzyme systems involved in the L-ascorbic acid biosynthesis in the fish are of the same nature as those occurring in mammalian liver (Salseduc, 1968). The conclusion may be that, as in the rat, in the fish also stimulation of the glucuronic acid system occurs during drug treatment.

The investigation of the glucuronic acid system got an impetus from the observation that stimulation of this system by drugs and the inherent increased excretion of L-ascorbic acid coincide with the induction of an increased activity of drug-metabolizing enzyme systems (Conney and Burns, 1959). This coincidence of the stimulating influence on drug metabolism and the stimulating influence on the glucuronic acid system could be confirmed for different groups of drugs (Table 1). The stimulating drugs are chemically and pharmacologically unrelated. It is not necessary that the stimulating compound is metabolized itself. Barbital, although very slightly metabolized (Ebert et al., 1964) is nevertheless a strong stimulator. Neither is it necessary that the drugs involved or metabolites of them are conjugated to a large extent with D-glucuronic acid (Burns et al., 1957).

The increased excretion of L-ascorbic acid in the urine after drug treatment was especially studied in the rat. The quantity of L-ascorbic acid excreted during treatment of the animals with various stimulating drugs (summarized in Table 1) amounts to 50-fold of the normal excretion. Certain groups of compounds, such as phenols, salicylates, sulphonamides and hormones are weak stimulators. Examples of drugs that do not stimulate the L-ascorbic acid excretion are the readily water-soluble compounds acetazolamide and 2,4-dinitrophenol

and the slightly water-soluble compounds caffeine and theobromine. Only if applied in relatively high doses, certain hormones bring about an increase in the L-ascorbic acid excretion, as a rule, however, not to a larger extent than the 2-fold of the normal excretion. As was reported for androgens and anabolic steroids by Vanha-Perttula (1963) and for the growth hormone by Salomon and Stubbs (1961) the stimulating effect of these compounds becomes manifest only after a few days of application. This in contrast to the stimulating effect of the drugs, mentioned in Table 1.

2.3 The biochemical basis of the stimulating action of drugs on the glucuronic acid system

2.3.1 Results obtained in in vivo experiments

L-ascorbic acid biosynthesis

In the investigations on the biochemical background of the stimulating action of drugs on the L-ascorbic acid biosynthesis emphasis was put on those drugs which act as strong stimulators. As such, the sedatives barbital and chloretone, the antipyretic aminophenazone and the carcinogen 3-methylcholanthrene can be mentioned. As said before, active stimulating drugs can increase the excretion of L-ascorbic acid by rats tremendously. Upon this fact Longenecker et al. (1940) based the following conclusion: 'In view of the fact that without any other L-ascorbic acid intake the quantities excreted each day are far in excess of the total body stores, the phenomenon obviously involves an increased rate of synthesis unless one postulates a normal, very rapid turnover that might be blocked by a second step with resultant accumulation of L-ascorbic acid'. By applying radio-active isotopes, it was possible to obtain definite proof for the increased biosynthesis. By these means, Burns et al. (1953) and Conney et al. (1961) determined the body pool, the turnover rate (expressed as the amount synthesized or the amounts metabolized and excreted each day) and the excretion of L-ascorbic acid in rats during treatment with the stimulating drugs chloretone, pentobarbital or 3-methylcholanthrene. In untreated rats the body pool was about 10 mg L-ascorbic acid per 100 g body weight; the amounts metabolized and excreted were found to be 2.0 mg and 0.5 mg per 100 g body weight per day respectively. Compared with the untreated animals, under the influence of drug treatment the body pool increased with a factor 2, the turnover rate with a factor 5 to 10, the quantity metabolized with a factor 5, and the quantity excreted with a factor 5 to 40.

After it was proved that as a result of the stimulation an increased biosynthesis of L-ascorbic acid takes place, the question arose at which step of the

sequence of enzymatic reactions leading from D-hexose to L-ascorbic acid, stimulation is initiated.

D-glucuronic acid biosynthesis

An indication of the D-glucuronic acid pathway (Fig. 2 and in Fig. 1: I and Ia) as the important sequence where initiation takes place was found in the observation of Burns et al. (1954) that application of barbital to rats not only results in an enhanced L-ascorbic acid excretion but also in an enhanced D-glucuronic acid excretion. These data and the observations that during treatment with the stimulating drugs barbital, chloretone and 3-methylcholanthrene an increased conversion of D-glucose-1-C^{14} into radio-active L-ascorbic acid as well as into radio-active D-glucuronic acid takes place (Burns and Evans, 1956; Conney et al., 1961), point to the glucuronic acid pathway as the system in which stimulation is initiated. It is shown by Evans et al. (1960) that D-galactose-1-C^{14} is incorporated into D-glucuronic acid, L-gulonic acid and L-ascorbic acid in barbital-treated rats, even to a higher degree than D-glucose-1-C^{14}. The labeled carbon atom of D-galactose undergoes considerably less randomization. So evidence is presented that D-galactose is a more direct precursor of the acids than is D-glucose. In view of the evidence that D-galactose is a more direct precursor of UDPG (Siu and Wood, 1959) than D-glucose, the results of the *in vivo* experiments given by Evans et al. (1960) are in accord with an enhanced carbohydrate metabolism via the nucleotides UDPG and UDPGA during stimulation. Chloretone does not stimulate the incorporation of D-glucurono-lactone-1-C^{14} and L-gulonolactone-1-C^{14} into L-ascorbic acid (Burns and Evans, 1956). These results suggest that the primary effect of chloretone and probably of stimulating drugs in general, is to increase the synthesis of D-glucuronic acid in the cell, which is then utilized for the synthesis of L-ascorbic acid.

The fact that both D-glucose and D-galactose are converted into L-ascorbic acid more rapidly under the influence of drug treatment indicates that the probable site of action is located in the enzymatic steps which the D-glucose route and the D-galactose route have in common (Fig. 7). The enzymes connected with these steps e.g. UDPG dehydrogenase, nucleotide pyrophosphatase, phosphatase, UDP glucuronyltransferase and β-glucuronidase (Fig. 2) require further attention in this respect. See further Section 2.3.2.

L-xylulose biosynthesis

During treatment of pentosuria patients with borneol, aminophenazone or phenazone, an increased excretion of L-xylulose in the urine is observed within 24 hours after the first dose (Enklewitz and Lasker, 1935). Aminophenazone

and phenazone strongly increase the L-ascorbic acid excretion in the rat; borneol does so to a lesser degree (Longenecker et al., 1940). The increased excretion of L-xylulose can be interpreted as the stimulating influence of borneol, aminophenazone and phenazone on the glucuronic acid pathway, similar to that observed for barbital, chloretone and 3-methylcholanthrene, which, as was indicated before, have as a primary effect an increase in the synthesis of D-glucuronic acid from D-glucose. In healthy individuals treated with drugs the increased activity in the xylulose pathway does not result in the appearance of extra pentose in the urine. In patients with pentosuria the pathway is blocked at the point where the conversion of L-xylulose to xylitol takes place (Section 1.1.4), so that in such patients the stimulation results in an increased excretion of L-xylulose in the urine.

D-glucaric acid biosynthesis

Since the stimulation of the glucuronic acid pathway, as mentioned before, results in an increased excretion of L-ascorbic acid and, under particular conditions, of L-xylulose, it might be expected that also an increased formation and excretion of another product of the glucuronic acid system, i.e. D-glucaric acid, will take place. Marsh and Reid(1963) obtained indications for this supposition. These investigators treated rats with barbital and chloretone and observed that in the urine obtained during drug treatment the reaction on D-glucaric acid is more positive.

The reaction on D-glucaric acid is based on the acid-potentiated inhibition of β-glucuronidase. It is known that heating of D-glucaric acid at 100 °C for 40 minutes at a pH of about 3 results in the formation of the D-glucaro-(1→4)-lactone, a strong inhibitor of the enzyme β-glucuronidase (Section 4.3.1.). There are no indications of other substances in the urine, which after such a treatment act as inhibitors for this enzyme. The acid-potentiated inhibition by urine is strongly increased after drug treatment. It is proposed that the increased excretion of D-glucaric acid in the urine after drug treatment is the result of an increased formation of this acid in the liver, based on a stimulation of the glucuronic acid pathway (Marsh and Reid, 1963).

Is *de novo* enzyme synthesis involved in the stimulation of the glucuronic acid system by drugs?

It has been put forward that the stimulation of the glucuronic acid pathway is the result of the enhanced level of hepatic enzymes involved in the formation of D-glucuronic acid. An argument for this supposition was the observation in rats treated with the stimulating drugs barbital and 3-methylcholanthrene that ethionine, an inhibitor of protein synthesis, prevents the response in the urinary

excretion of L-ascorbic acid (Touster et al., 1960; Neumann et al., 1963; Hollmann and Touster, 1962). However, the prevention was only found to be partial (de Matteis, 1964; Kato, 1967). Available evidence now indicates that the inhibition of protein synthesis in the liver by ethionine is a secondary result of a decrease in the concentration of ATP (Villa Trevino et al., 1963). This decrease would also explain the strong decrease in the levels of D-fructose-1,6-diphosphate, D-glucose-6-phosphate and NAD after ethionine treatment (Bartels and Hohorst, 1963; Stekol, 1963). The effect of ethionine on the response to drugs could then be explained from the decreased availability of glycolytic intermediates and NAD for the conversion into UDPGA, rather than from inhibition of enzyme synthesis. The observation of de Matteis (1964) that actinomycin D does not inhibit the stimulating effect of 2-allyl-2-isopropylacetamide on L-ascorbic acid excretion by rats indicates that *de novo* enzyme synthesis is not involved in the stimulation.

Involvement of hormones in the stimulation of the glucuronic acid system

Because of the apparent lack of a relationship between chemical structure or pharmacodynamic activity and the capacity of drugs to enhance the L-ascorbic acid excretion, Burns et al. (1957) considered the possibility that the drugs might act in an unspecific indirect way. Their finding that in hypophysectomized rats no stimulation of the L-ascorbic acid excretion takes place after chloretone or barbital treatment points in the direction of certain hormonal control processes as possible sites of initiation. These results, however, could not be confirmed by other investigators (Salomon and Stubbs, 1961; Hollmann and Touster, 1961; Klinger et al., 1965). The observations, however, still leave open the possibility of the stimulation of the glucuronic acid system by the action of the drugs via certain hormonal processes. In Section 7.1.1 these aspects will be discussed in further detail and extended with the results of new experiments.
The fact that in nephrectomized rats during drug treatment the level of L-ascorbic acid in the blood is strongly increased (Burns et al., 1960; Martin, 1961) indicates that renal mechanisms are not involved.

2.3.2 Results obtained in in vitro experiments

It is a plausible assumption that the increased excretion of the products of the glucuronic acid system is the result of an enhanced synthesis of these products in the liver. A convincing argument for this point of view is found in the fact that in mammals the liver is the organ where L-ascorbic acid is synthesized (Chatterjee et al., 1961). Data for direct experimental proof for the enhanced biosynthesis of the various products of the glucuronic acid system in the perfused liver of drug-treated animals were not found in the literature.

Effect of stimulating drugs on enzyme activity

Efforts to stimulate the L-ascorbic acid synthesis in liver preparations were unsuccessful up to now. The application of stimulating drugs to liver homogenates gave no indication whatsoever of an increased activity of the enzymes involved in the glucuronic acid system, nor of an enhanced L-ascorbic acid synthesis. In fact the stimulating drugs such as chloretone and barbital are able to inhibit certain of the enzymes involved in the glucuronic acid system. The enzyme glucuronolactone reductase is found to be inhibited non-competitively by barbital (Mano et al., 1961). The formation of D-glucuronic acid-1-phosphate from UDPGA is also inhibited by barbital (Hollmann and Touster, 1962). The enzyme D-glucuronolactone dehydrogenase is inhibited non-competitively by barbital, pentobarbital, thiopental and chloretone (Marsh, 1963c). The concentrations of the various drugs which cause an enzyme inhibition *in vitro* are of the same order as those occurring in the plasma of the animals during the stimulation experiments. As a matter of fact the effectiveness of the drug concentrations concerned in *in vitro* and in *in vivo* experiments is not necessarily comparable. The results mentioned might, however, indicate that the enzymatic steps inhibited by the direct action of the various drugs are probably not rate-limiting as far as the conversions in the glucuronic acid system are concerned.

The information about the influence of drugs on the breakdown of L-ascorbic acid in liver tissue is not complete. It has been shown by Conney et al. (1961) that the stimulating drugs 3-methylcholanthrene, chloretone and pentobarbital *in vivo* markedly shorten the half-life time of L-ascorbic acid in the rat. Koch and Klinger (1963) report that in liver homogenates of rats, which were treated for 4 days with barbital, a retarded disappearance of added L-ascorbic acid takes place, although the L-ascorbic acid level originally present in the homogenate does not decrease under the same experimental conditions. They report an accelerated disappearance of exogenous as well as endogenous L-ascorbic acid after treatment with hexobarbital. Taking into account the small number of experiments performed, it is doubtful whether definite conclusions can be drawn from the experiments of Koch and Klinger.

UDPG dehydrogenase

In Section 2.3.1 the possibility that the stimulation of the glucuronic acid system by drugs is based on an increased synthesis of enzymes has been put forward. Application of drugs to liver tissue preparations as was described before, does not result in any stimulation. The study of enzyme activity in the liver preparations of animals treated with drugs gave interesting results. Conney et al. (1961) reported that in liver homogenates of rats treated orally with chloretone for 7 days an increased conversion of D-galactose-1-C^{14} to D-

glucuronic acid-1-C^{14} takes place. In more detailed studies on the effect of treatment with chloretone on the formation of D-glucuronic acid and L-ascorbic acid by liver homogenates an enhanced activity of the enzyme UDPG dehydrogenase was reported by these investigators. Neither the activities of liver microsomal pyrophosphatase and phosphatase, the latter tested with D-glucuronic acid-1-phosphate as a substrate, nor the activities of the enzymes involved in the conversion of D-glucuronolactone and L-gulonolactone into L-gulonolactone and L-ascorbic acid respectively, appeared to be increased after treatment with chloretone. The activity of L-gulonolactone oxidase was even found to be decreased. This might indicate that the increased activity of UDPG dehydrogenase — the only activity found to be enhanced after pretreatment with chloretone — is primarily involved in the stimulation of the glucuronic acid pathway by drugs. Hollmann and Touster (1962) also described an increased activity of UDPG dehydrogenase in liver homogenates from guinea pigs and rats treated during 4 days with chloretone or barbital. Zeidenberg et al. (1967) observed that in rats treated daily with phenobarbital the increased activity of the UDPG dehydrogenase does not manifest itself clearly before the treatment has lasted for 3 or more days. Under the same circumstances the activity of UDP glucuronyltransferase was also found to be enhanced after only a few days of treatment. As will be discussed in more detail in the experimental sections (see also Aarts, 1966), pretreatment of rats or guinea pigs with barbital for a short period, 1 to 3 days, did not result in an increase in the activity of UDPG dehydrogenase in the liver homogenates.

UDP glucuronyltransferase

The possible significance of the increase of the activity of the UDPG dehydrogenase after drug treatment for the stimulation in the glucuronic acid system cannot be generalized, since for certain stimulating drugs such as aminophenazone and the polycyclic carcinogens, no increase in the activity of the enzyme concerned could be detected (Inscoe and Axelrod, 1960; Touster et al., 1961). These drugs, however, were found to be able to increase the activity of UDP glucuronyltransferase.

The UDP glucuronyltransferase is involved in the transfer of D-glucuronic acid from the donor UDPGA to the acceptor drugs and drug metabolites under the formation of glucuronides (Section 1.1.5). It has been put forward that the increased activity of UDP glucuronyltransferase might also lead to an enhanced formation of D-glucuronic acid and L-ascorbic acid from UDPGA (Touster et al., 1962). As discussed in Sections 1.1.1 and 1.1.5 it is questionable whether an UDP glucuronyltransferase is involved in that part of the glucuronic acid system, which leads from UDPGA to D-glucuronic acid.

From the foregoing it is clear that the definite proof of the significance of increased activities of UDPG dehydrogenase and UDP glucuronyltransferase for the stimulation of the glucuronic acid system has not been established. Further investigations in this respect are required (Section 5.4 and Chapter 6).

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

Wistar rats and guinea pigs maintained on Hope Farms Laboratory Diet, with free access to water were used. The guinea pigs received an extra supply of 20 mg L-ascorbic acid per day; the rats received L-ascorbic acid-free food.

3.2 Analytical methods

Preparation and storage of tissue

Immediately after killing the animals by a blow on the head, the tissues to be used were removed, cooled in ice and homogenized with the Ultra-turrax homogenizer TP 18/2. The homogenized tissue was kept at 4° if it was to be used without delay and stored at a temperature below 0° if it was to be used later.

Preparation and storage of urine

The urine for determination of L-ascorbic acid was collected in a 10 % oxalic acid solution. For other determinations urine was collected as such. Urine was stored at 4° or below 0° depending on the period of time before analysis (see above).

L-ascorbic acid in urine

L-ascorbic acid was determined by the 2,6-dichlorophenolindophenol method (Olliver, 1954).

L-ascorbic acid in tissue

The method of Schaffert and Kingsley (1955) was applied.

D-glucaric acid in urine

D-glucaric acid was assayed with the aid of the inhibiting action of its (1→4)-lactone on β-glucuronidase (Section 4.3.1). In the assay phenolphthalein glucuronide is used as substrate. If the D-glucaric acid content has to be assayed in urine which contains amounts of glucuronides coming from drugs or drug metabolites an interference in the D-glucaric acid assay may occur. For that reason the following procedure was followed if glucuronides were expected to be present in the urine. The urine was adjusted to pH 7 and heated at 100° for 20 min. After readjustment at pH 7, if necessary, the urine was incubated with β-glucuronidase (Sigma type I, active at pH 7, 1000 U/ml urine) for

48 hours at 37° to eliminate possibly interfering glucuronides. After inactivation of the β-glucuronidase by heating for 5 min at 100°, the urine was extracted twice with a double volume of ethyl acetate and assayed by the procedure of Marsh (1963a).

The method given above was applied if patients or animals had been treated with the following drugs: thiopental, aminophenazone, phenylbutazone, phenytoin, tolbutamide, borneol and salicylic acid.

If very little glucuronide was expected to be present in the urine, as is the case for barbital, nikethamide and DDT, the urine was adjusted to pH 7 and heated to 100° for 20 min. Then the urine was extracted twice with a double volume of ethyl acetate and assayed by the procedure of Marsh(1963a).

The urines of control animals and drug-treated animals were treated in the same way.

D-glucaric acid in tissue

The tissue homogenate was centrifuged at 9000 *g* for 20 min. The supernatant was deproteinized and a suitable portion was used for the D-glucaric acid assay as described by Marsh(1963b).

D-glucose in urine (qualitative)

The color test with Clinistix reagent strips was used.

D-glucuronic acid in urine

Dische's carbazole reaction modified by Yuki and Fishman (1963) for differential analysis was applied.

Glucuronides in urine

Dische's carbazole reaction modified by Yuki and Fishman(1963) was applied.

D-mannaric acid in urine

The total quantity of D-glycaric acids (in this case D-mannaric acid plus D-glucaric acid) was measured with the method of Ishidate et al.(1965). The quantity of D-glucaric acid in urine was determined as described above. The quantity of D-mannaric acid was calculated by subtracting the quantity of D-glucaric acid from the total quantity of D-glycaric acids determined.

3.3 Enzymatic assays

Immediately after killing the animal the liver was removed, cooled in ice and homogenized in 4 volumes of 0.15 M KCl in a Potter-Elvehjem grinder. Homogenates were centrifuged for 30 min at 9000 *g* and the supernatants were used for all assays.

Demethylation

The rate of demethylation was determined with the procedure described by Smith et al. (1963) with slight modifications. Glucose-6-phosphate dehydrogenase (5 Kornberg units of G 6-OH 15303 Boehringer) was added, aminophenazone was used as a substrate, and the incubation time was 1 or 3 hours.

D-glucuronolactone dehydrogenase

D-glucuronolactone dehydrogenase was determined according to Marsh (1963b).

Tyrosine aminotransferase

Tyrosine aminotransferase was determined according to Kenney (1959) except that no pyridoxal phosphate was added during incubation. Under the circumstances in the course of this study the addition of the compound is not essential.

UDPG dehydrogenase

UDPG dehydrogenase was determined according to Strominger et al. (1957).

Proteins

Proteins were estimated by the procedure of Lowry (1951), using bovine serum albumin (Sigma) as a standard.

CHAPTER 4

THE INFLUENCE OF DRUGS ON THE EXCRETION OF D-GLUCARIC ACID IN MEN AND GUINEA PIGS

4.1 Introduction

The stimulation of the glucuronic acid system by drugs becomes especially manifest in an increased excretion of L-ascorbic acid in the animals treated. The question arises whether the stimulation after drug treatment also occurs in man. As known, man lacks the ability to synthesize L-ascorbic acid (Section 1.1.2.1) and therefore an increase in the excretion of this acid cannot be expected and cannot serve as an indication of the stimulation under discussion.

The observation of the increased excretion of L-xylulose in patients with pentosuria after treatment with drugs such as borneol, aminophenazone and phenazone indicates that also in man drug treatment results in a stimulation of the glucuronic acid system; however, in healthy individuals this stimulation will not manifest itself by an increased excretion of L-xylulose in the urine, since then the L-xylulose formed is processed further in the glucuronic acid cycle (Section 1.1.4). In order to detect a stimulation of the glucuronic acid system in man the endproduct of one of the other pathways should be taken into consideration.

The question arises whether D-glucuronic acid can be considered as an endproduct. The acid is normally excreted in the urine of mammals. Although it is excreted in greater quantities after treating rats or guinea pigs with a stimulating drug such as barbital (Burns et al., 1957), it is still questonable whether the increased excretion of D-glucuronic acid is suitable as a criterion for the enhanced activity in the glucuronic acid system.

According to the results of balance experiments (Südhof and Schellong, 1953; Fretwurst and Ahlhelm, 1953) and experiments with radio-active products (Butler and Packham, 1955), D-glucuronolactone administered orally to mammals can be metabolized almost completely, so that practically no D-glucuronic acid administered in the lactone form appears in the urine. The products formed from D-glucuronolactone are L-ascorbic acid, D-glucaric acid, glycogen, CO_2 and H_2O (Sections 1.1.2, 1.1.3, 1.1.4). D-glucuronolactone administered to patients with hepatitis or liver cirrhosis and to dogs with an Eck fistula is metabolized to a lesser degree than under normal conditions (Hollmann, 1954). The latter data indicate that the metabolism of D-glucuronolactone or D-

glucuronic acid primarily takes place in the liver (Hollmann, 1964). As for the D-glucuronolactone and D-glucuronic acid formed in the liver, it may also be expected that in the normal liver they are easily converted into L-ascorbic acid, D-glucaric acid and glycogen so that only a small fraction of them may appear in the urine.

If D-glucuronic acid as such or as a constituent of food is applied orally to mammals a definite excretion of the acid in the urine is observed. Exogenous D-glucuronic acid as opposed to exogenous D-glucuronolactone, probably because of its high polarity, is not or only slightly taken up by the liver (Eisenberg, 1957) but excreted quickly by the kidney. The varying quantities of D-glucuronic acid in the urine after intake of different kinds of food (Remmer, 1959; Hänninen, 1966) can be explained by the varying quantities of D-glucuronic acid in the food.

Another reason why the D-glucuronic acid excretion is less suitable as a criterion for the stimulation of the glucuronic acid system is the fact that many drugs and their metabolic products are conjugated to D-glucuronic acid. The conjugated forms (glucuronides) are excreted with the urine or bile. The quantities of these glucuronides are mainly dependent on the quantity of acceptor (drugs or drug metabolites) available for the UDPGA. Taking into account the unstable character of these glucuronides and the presence of β-glucuronidase in the gut and in the urine, it is difficult to differentiate between the D-glucuronic acid in the urine originating from glucuronide disjunction or that originating directly from the glucuronic acid pathway in the liver.

The question arises whether the glucuronide excretion as such can serve as a criterion. The biochemical link between the glucuronidation of drugs and drug metabolites and the rate of turnover in the glucuronic acid system is in no way clear as yet (Section 1.1.5). For the glucuronide formation the presence of suitable acceptors such as drugs and drug metabolites is of predominant significance. This implies that the glucuronide excretion cannot serve as a suitable parameter for the activity in the glucuronic acid system.

Another endproduct of the glucuronic acid system to which little attention has been paid in the past, viz. D-glucaric acid, may offer a better opportunity. The conversion of D-glucuronic acid to D-glucaric acid takes place via D-glucuronolactone as an intermediate. Under normal conditions D-glucaric acid is excreted by man in a quantity of about 10 mg per 24 hours. D-glucuronolactone, applied orally to man is converted partially to D-glucaric acid (Marsh, 1963a; Okada et al., 1964). A dosis of 5 g D-glucuronolactone results in an excretion of about 1 g D-glucaric acid in the urine. On the basis of these data it may be expected that an enhanced metabolism via D-glucuronic acid during stimulation of the glucuronic acid system, may as well result in an enhanced formation and an attendant excretion in the urine of D-glucaric acid. Up to

now little attention has been paid to the formation and excretion of D-glucaric acid under the influence of drugs. More extensive investigation of this aspect of the drug-induced stimulation of the glucuronic acid system is required.

4.2 The influence of barbital on the formation and the excretion in the urine of D-glucaric acid in guinea pigs

4.2.1 The influence of barbital on the urinary excretion of D-glucaric acid in guinea pigs

Animal experiments can serve as a basis for the studies aimed at the investigation of the excretion of D-glucaric acid in patients. For this purpose the guinea pig is the most suitable animal since besides man and monkey, it is the only mammal known to lack the ability to synthesize L-ascorbic acid (Section 1.1.2.1). Barbital is chosen as a stimulating drug, since this compound has been used in many studies concerning the glucuronic acid system, especially with respect to the formation and excretion of L-ascorbic acid in the rat.

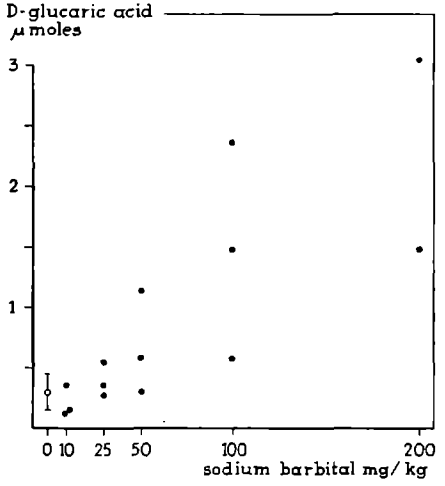

FIG. 8 *Effect of cumulative doses of barbital on the urinary excretion of D-glucaric acid in guinea pigs.*

A group of 14 female guinea pigs, weighing 300–400 g, was used. The animals were injected with saline i.p. at zero time, and sodium barbital i.p. at 24 hr. Urine was collected in two consecutive 24-hr periods. The individual D-glucaric acid excretions after barbital treatment are represented by closed circles; mean and S.D. of the D-glucaric acid excretions before barbital treatment are represented by an open circle with vertical line. A positive trend between the increase in D-glucaric acid excretion and the dose of barbital is found (Terpstra trend test, two-sided; P value < 0.001).

First the influence of various doses of barbital applied intraperitoneally (i.p.) on the D-glucaric acid excretion was studied. As shown in Fig. 8 a higher dose of the drug results in an increase in the quantity of D-glucaric acid excreted. Then the time course of the increased D-glucaric acid excretion after a single dose of barbital was investigated. On the basis of the result reported in Fig. 8, a dose of sodium barbital of 150 mg/kg was chosen. Already within 24 hours after application of the drug the D-glucaric acid excretion increased (Fig. 9). The increase is still significant on the 4th day after application and gradually levels off to the original value.

As mentioned before, both the enhanced excretion of L-ascorbic acid and the enhanced activity of the drug-metabolizing enzyme systems in the liver take place in the drug-treated rat (Table 1). The drug-metabolizing enzyme systems

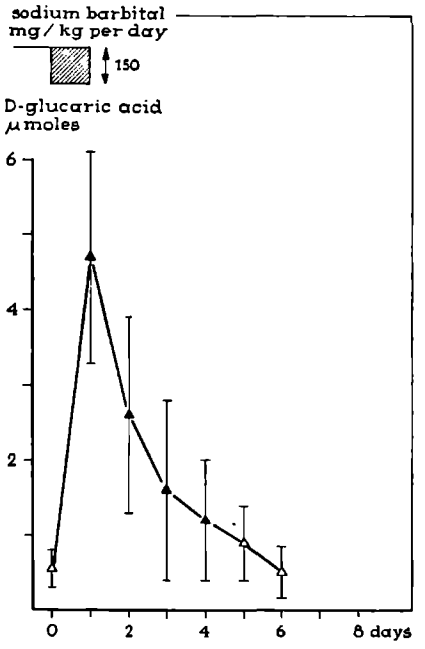

FIG. 9 *Effect of a dose of barbital on the urinary excretion of D-glucaric acid in guinea pigs.*

A group of 8 female animals, weighing 300–400 g, was injected with saline i.p. and after 24 hr with sodium barbital i.p. Urine was collected in 24-hr periods. Means with S.D. are shown. The urines of the 24-hr periods after treatment were compared with that of the 24-hr period before treatment. Significant differences are shown by closed symbols ($P \leq 0.05$; Wilcoxon signed-rank test).

involved are bound to the endoplasmic reticulum. They belong to the group of mixed-function oxidases, which need as co-factors $NADPH_2$ and oxygen. The terminal step in this oxidizing system is formed by the cytochrome P-450 (Ernster and Orrenius, 1965).

In an orientating experiment the effect of barbital upon the excretion of D-glucaric acid in the urine as well as upon the activity of liver tissue of metabo-

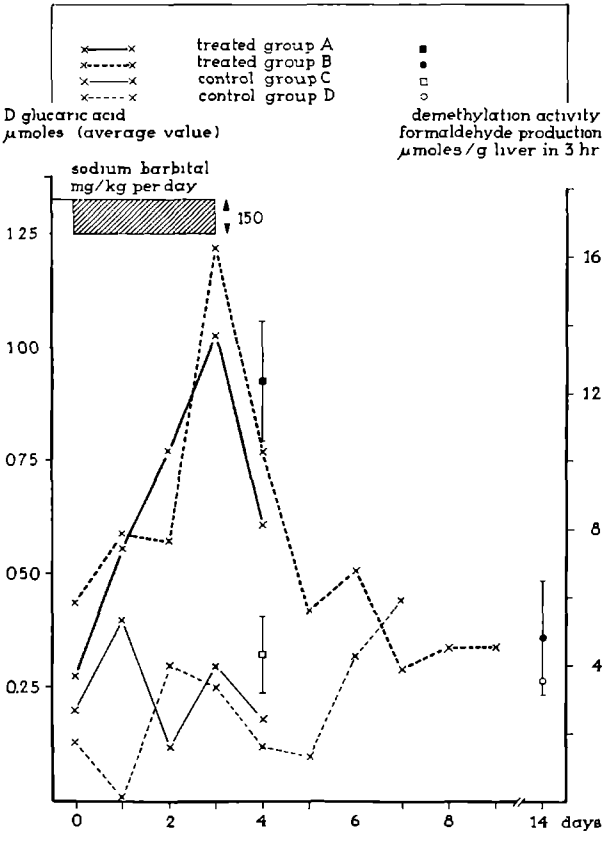

FIG. 10 *Excretion of D-glucaric acid in urine and demethylation activity in the liver of guinea pigs treated with barbital.*

Female animals, weighing 230–280 g, were used. Animals of each group A and B (5 and 5 respectively) were injected with sodium barbital 75 mg/kg i.p. twice daily during the 1st, 2nd and 3rd day; animals of groups C and D (5 and 3 respectively) were injected with saline i.p. Groups A and C were killed after the 4th day, groups B and D after the 14th day and the livers of each group were homogenized for estimation of the demethylation activity in each liver supernatant (9000 *g*).
The activity was tested with aminophenazone as a substrate.

lizing drugs was measured in guinea pigs. For the measurement of the drug-metabolizing activity the demethylation of aminophenazone by liver homogenate was measured. As shown in Fig. 10, the D-glucaric acid excretion in the urine as well as the demethylation activity in the liver increased after treatment of the guinea pigs with barbital.

4.2.2 Experiments to study the relation between the enhanced excretion of D-glucaric acid in the urine and the stimulation of the glucuronic acid system in the liver

D-glucuronic acid synthesized to a greater extent in the liver is the most probable source for the enhanced formation and excretion of D-glucaric acid. The formation and excretion of D-glucuronic acid is known to be enhanced after drug treatment (Chapter 2). However, theoretically there are other possible causes of an enhanced D-glucaric acid excretion.

It might be that the increased excretion of D-glucaric acid after drug treatment is the result of a depletion of a D-glucaric acid pool in the organism, or the result of an inhibition of the catabolic degradation of D-glucaric acid caused by the drug treatment. If the total body pool of D-glucaric acid in the animal (guinea pig) is compared with the extra quantity of D-glucaric acid excreted under the influence of the drug treatment, it is found that this quantity per period of 24 hours is significantly greater than the body pool (Table 2).

TABLE 2 *Body pool of D-glucaric acid in untreated guinea pigs, and the amount of D-glucaric acid in 24-hr urine of untreated and barbital-treated guinea pigs.**

	D-glucaric acid μmoles	
	body pool	urinary excretion in 24 hr**
Untreated (5)	0.38 ± 0.28	
Treated (6)		
before treatment		0.48 ± 0.10
after treatment		3.2 ± 1.6
		increase: 2.8 ± 1.6

* Of a group of 11 female guinea pigs, weighing 200–240 g, 6 animals were given single injections of sodium barbital 150 mg/kg i.p., and 5 animals were used for determination of the body pool of D-glucaric acid.

** Urine collection was started 24 hr before injection and immediately after injection. Means with S.D. are presented.

Note The increase in the D-glucaric acid excretion (per 24 hr) is higher than the body pool ($P = 0.03$; two-sided Wilcoxon test).

Definite proof for the absence of an inhibition of D-glucaric acid catabolism after drug treatment could not be given; however, a variety of experimental arguments could be obtained pleading for the direct relation between the enhanced carbohydrate metabolism via D-glucuronic acid in the liver during drug treatment and the enhanced formation and excretion of D-glucaric acid. D-glucuronolactone dehydrogenase involved in the conversion of D-glucuronolactone to D-glucaric acid is found to be present in the liver of mammals. The activity of this enzyme in the liver is high as compared to that in the other tissues. For the rat the presence of the enzyme was detected in liver, kidney and testis (Section 1.1.3). In other tissues such as uterus, thyroid gland, lung, duodenum and in plasma no significant activity was found. The enhanced excretion of D-glucaric acid in the urine after barbiturate treatment of rats, reported by Marsh and Reid (1963) could be confirmed, while it was also shown that after this treatment the D-glucaric acid level in liver tissue increased (Table 3). As reported in Chapter 2, definite proof is available for the enhanced

TABLE 3 *Effect of barbital treatment* on the amount of D-glucaric acid in rat liver.*

	D-glucaric acid/g liver mμmoles	P value
Treated:		
9 hr after injection of drug	25.9 ± 5.8 (6)	<0.01
20 hr after injection of drug	9.7 ± 6.8 (6)	>0.10
Control:	4.1 ± 1.6 (6)	

* Of a group of 18 female rats, weighing 180–200 g, 12 animals were given single injections of sodium barbital 150 mg/kg i.p. at zero time. The control animals were given saline at zero time and killed at 9 hr. The treated animals were killed at 9 hr or at 20 hr.
Means with S.D. are presented. Numbers in parenthesis indicate number of animals on which mean is based. P values were obtained by applying the two-sided Wilcoxon two-sample test.

synthesis of L-ascorbic acid in the liver of rats after treating them with drugs. This implies that after barbital treatment the L-ascorbic acid (Klinger and Koch, 1965a) as well as the D-glucaric acid content of the liver increased (Table 3). Taking into account the data that D-glucuronolactone is metabolized mainly in the liver (Section 4.1) and that D-glucuronolactone dehydrogenase occurs mainly in the liver, while in barbital-treated rats the D-glucaric acid level in the liver is strongly enhanced, it is likely that the D-glucaric acid synthesis in the liver is responsible for the enhanced level of D-glucaric acid in the

urine as caused by barbital. As will be shown later, in man barbiturate treatment also results in an enhanced excretion of D-glucaric acid in the urine. The activity of D-glucuronolactone dehydrogenase in human liver is high, while it is very low in the human kidney (Section 1.1.3). Also on the basis of these various data it is feasible that in man as well as in rat and guinea pig the increase in the D-glucaric acid excretion after drug treatment is based on an enhanced synthesis of it in the liver.

TABLE 4 *D-glucuronolactone dehydrogenase activity of guinea pig liver homogenates at various times after administration of barbital.*

Time after administration*	D-glucuronolactone dehydrogenase activity per mg protein 10^2 μmoles/hr		
hr	control	treated	P value
5	3.4 ± 0.9 (6)	2.7 ± 0.9 (6)	>0.25
9	2.8 ± 1.6 (6)	4.2 ± 1.6 (6)	>0.10
24	3.4 ± 0.8 (6)	4.9 ± 2.6 (11)	>0.10
72	3.0 ± 0.7 (6)	2.7 ± 1.0 (6)	>0.25

* Female guinea pigs, weighing 300–400 g, were given single injections of sodium barbital 150 mg/kg i.p. at zero time. The 72-hr group were given three injections: at zero time, at 24 hr and at 48 hr; the control animals were given saline.
The figures and P values are calculated as indicated in Table 3.

Taking into account the high probability that the increased levels of D-glucaric acid in the liver and in the urine after treatment with barbiturates are due to an enhanced formation of this acid via D-glucuronic acid in the liver, the following question arises. Is this enhanced formation due to a drug-induced enhanced formation of D-glucuronic acid resulting again in an enhanced metabolism via the glucaric acid pathway, as is proposed by Marsh and Reid (1963), or to an enhanced utilization of D-glucuronic acid based on a stimulating effect of barbital in the glucaric acid pathway? As shown in Table 4, the D-glucuronolactone dehydrogenase activity in the liver of guinea pigs is not found to be increased after drug treatment. In Table 5 the influence of barbital on the *in vivo* conversion of D-glucuronolactone into D-glucaric acid is presented. It may be seen that during barbital treatment no increase of the D-glucaric acid excretion is observed in D-glucuronolactone-loaded rats. The dose of D-glucuronolactone is chosen so high that it largely surpasses the concentration of this substrate in the liver under normal conditions or during drug treatment. This results, as is shown, in a strong increase in the D-glucaric acid excretion in the urine and therefore in a strong increase of its formation.

TABLE 5 *Influence of barbital on the urinary excretion of D-glucaric and D-mannaric acid in guinea pigs treated with D-uronolactones.**

Compound	Oral dose	Urinary excretion — μmoles/12 hr		
		D-glucaric acid		
	μmoles	without barbital	with barbital	P value
D-glucuronolactone	0	0.12 ± 0.05 (6)	0.45 ± 0.21 (8)	<0.01
„	300	19.1 ± 7.7 (7)	17.3 ± 2.4 (7)	>0.25
„	750	42.8 ± 14.9 (6)	43.0 ± 11.3 (6)	>0.25
„	1600	101.8 ± 31.7 (7)	101.6 ± 19.9 (7)	>0.25
D-mannuronolactone	660		0.39 ± 0.24 (5)	
		D-mannaric acid		
„	660	33.6 ± 4.4 (5)	29.8 ± 7.1 (5)	>0.25

* Male guinea pigs, weighing 250–300 g, were given single injections of sodium barbital 150 mg/kg i.p. at zero time. The animals were given twice D-glucuronolactone: at zero time and at 6 hr. D-mannuronolactone was given in a single dose. The control animals were given saline i.p.
The figures and P values are calculated as indicated in Table 3.

These experiments (Tables 4 and 5) give no indication whatsoever of an enhancement under the influence of barbital in the conversion of D-glucuronolactone into D-glucaric acid, the second step in the conversion of D-glucuronic acid into D-glucaric acid.

This conclusion is further confirmed by studying the conversion of D-mannuronolactone into D-mannaric acid. It has been shown by Sadahiro et al. (1966) that also in this conversion D-glucuronolactone dehydrogenase very probably is involved (Section 1.1.3). No enhancement is found for the conversion of D-mannuronolactone into D-mannaric acid in the guinea pig during barbital treatment (Table 5).

As may be seen from Table 5, the capacity to convert D-glucuronolactone to D-glucaric acid is very high. After application of high doses of D-glucuronolactone the D-glucaric acid excretion in the urine is about 100 μmoles per 12 hours, while without application these quantities are smaller than 1 μmole. During barbital treatment the quantity excreted per 24 hours is 1 to 5 μmoles (Tables 2 and 5; Figs. 8, 9 and 10). This indicates that under normal conditions and even after barbital treatment only a small fraction of the conversion capacity is used, so that an enhancement of this capacity during drug treatment will only mean an even greater increase of a tremendous reserve capacity already present.

4.3 The urinary excretion of D-glucaric acid in men after the use of drugs

The amount of barbiturate per kg body weight given to guinea pigs in the experiments such as described (Section 4.2) is generally very much higher than the clinical dose used in man. One of the reasons for this difference is the fact that experimental animals, especially the smaller species such as mouse, rat and guinea pig, tend to eliminate drugs more rapidly than humans. The question arises whether also the relatively low clinical therapeutic doses used in man also cause a stimulation of the glucuronic acid system. For this purpose the urinary excretion of D-glucaric acid in humans after the use of suitably chosen drugs can be studied. The chosen drugs such as thiopental, phenobarbital, phenytoin, phenylbutazone and aminophenazone, are known to cause an enhanced formation of L-ascorbic acid in experimental animals. In addition the urine of diabetic patients treated with tolbutamide and the urine of volunteers taking salicylic acid were analyzed. Tolbutamide and salicylic acid enhance the excretion of L-ascorbic acid in rats to a much lower degree than the drugs mentioned earlier.

TABLE 6 *Urinary excretion of D-glucaric acid in patients* undergoing thiopental anaesthesia.*

	D-glucaric acid mg/24-hr urine	P value
Before first thiopental injection	6.5 ± 5.7	0.03
During and after thiopental anaesthesia	22.3 ± 18.9	

* Orthopoedic operations in 11 patients were concerned. As premedication was given atropine (0.25–0.50 mg), meperidine (50 mg) and promethazine (50 mg) s.c. The total amount thiopental given varied from 500 tot 800 mg. Anaesthesia proceeded for 2.5 to 4.0 hr.
Means with S.D. are presented. The P value was obtained by applying two-sided Wilcoxon signed-rank test.

In the study on the influence of thiopental the D-glucaric acid level was determined in the two 24-hr urine portions produced before and after administration of the first dose (Table 6). The later samples were found to contain higher levels of D-glucaric acid. Since the urine was obtained from patients treated surgically the possibility should be considered that this rise is not only caused by the thiopental, but may be partly due to the premedication. In the study on the influence of the other drugs mentioned a comparison with urine collected before treatment was not possible since the patients had already been under drug treatment for

TABLE 7 *Effect of drugs on the urinary excretion of D-glucaric acid in human subjects.**

Drug administered, daily dose	Group	Period of drug administration	D-glucaric acid mg/24-hr urine	P value
Aminophenazone, 1800–2400 mg	polyarthritis rheumatica	two weeks or longer	39.1 ± 23.4 (10)	<0.001
Phenylbutazone, 400–600 mg	polyarthritis rheumatica	two weeks or longer	11.3 ± 3.9 (10)	<0.01
None	polyarthritis rheumatica		8.7 ± 2.6 (6)	0.22
Phenobarbital and phenytoin, 100–300 mg and 90–225 mg	epilepsy	two months or longer	62.5 ± 38.6 (14)	<0.001
Tolbutamide, 500–2000 mg	diabetes	two months or longer	5.5 ± 3.2 (10)	>0.25
Salicylic acid, 1500 mg	healthy	10 days	5.5 ± 2.0 (5)	>0.25
None	healthy		6.5 ± 3.3 (17)	

* The figures and P values are calculated as indicated in Table 3.

some time; thus the urine of healthy persons (men and women[1]) was used to obtain reference values for the D-glucaric acid excretion (Table 7). In patients treated with aminophenazone, phenylbutazone or with a combination of phenobarbital and phenytoin the D-glucaric acid excretion was found to be higher than in the controls. The interpretation of these data is complicated by the possibility that the enhanced excretion might be influenced by the condition of the patient. The patients treated with aminophenazone and phenylbutazone were suffering from polyarthritis rheumatica. A group of patients with the same disease in the same hospital but not subjected to a treatment with drugs did not show an enhanced excretion of D-glucaric acid. It may be concluded therefore, that polyarthritis rheumatica is not involved in this phenomenon. As for the epileptics treated with phenobarbital and phenytoin it seems rather improbable that their disease is a factor in an enhanced excretion of D-glucaric acid.

During the intake of salicylic acid by healthy persons no change in the D-glucaric acid excretion was observed. The same holds for diabetics treated with tolbutamide. Tolbutamide is known to be oxidized to a carboxylic acid in the liver. This product has a hydrophilic character and is conjugated easily, so that

[1] Marsh (1963a) reports that the D-glucaric acid excretion in man is about 20% higher than in woman, but from his data it cannot be concluded whether this difference is significant. In our experiments no significant difference between the D-glucaric acid excretion by healthy man and woman was observed.

it is eliminated rapidly. The same holds for salicylic acid. It seems possible therefore, that salicylic acid and tolbutamide do not cause an enhanced D-glucaric acid excretion since the therapeutic doses were not sufficient to reach a concentration high enough to stimulate the glucuronic acid system. The half-life time in man for salicylic acid and tolbutamide is 5 to 6 hours (Südhof et al., 1958; Levy, 1963). The half-life time for aminophenazone, phenylbutazone and phenobarbital, drugs which cause an enhanced D-glucaric acid excretion, is 3 to 6 hours, 72 hours, and 48 to 96 hours respectively (Brodie and Axelrod, 1950; Burns et al., 1953). In the case of aminophenazone, besides the drug itself, the metabolite 4-aminoantipyrine may also have a stimulating effect on the glucuronic acid system.

Another possibility might be that an enhancement of the D-glucaric acid formation requires an excessive stimulation of the glucuronic acid pathway (Section 5.3.2). For the latter point of view argues the fact that even high doses of salicylic acid do not cause an enhanced D-glucaric acid excretion in the rat (Table 13) while the administration of salicylic acid to this animal is shown to cause only a slightly enhanced excretion of L-ascorbic acid (Longenecker et al., 1940). As will be shown, after administration of tolbutamide in the rat a slightly enhanced excretion of L-ascorbic acid can be observed too, while an enhanced D-glucaric acid excretion is not observed (Table 10).

4.3.1 Definite proof for the enhanced D-glucaric acid excretion in man after drug treatment by means of a semi-quantitative isolation of urinary D-glucaric acid

The enzymatic determination of D-glucaric acid used in this study (Marsh, 1963a) is based on the inhibition of β-glucuronidase by the (1→4)-lactone of D-glucaric acid (Fig. 11). In this determination one part of the urine is heated after acidification and another part after neutralization. In the first sample about 30 % of the D-glucaric acid present is converted into the (1→4)-lactone, in the other sample the (1→4)-lactone, if present at all, will be converted into the acid. The difference in inhibitory action between the acid-treated and neutralized urine fraction is a measure for the amount of D-glucaric acid present. The potentiation by acid treatment is essential for the specificity of this method. Apart from D-glucaric acid, D-galactaric acid is the only substance naturally occurring known to show this acid-potentiated inhibition. However, an amount of D-galactaric acid, a 50-fold larger than that of D-glucaric acid, is needed in order to get the same degree of inhibition (Levvy, 1952) (Fig. 11). Moreover, Marsh (1963a) and Okada (1964) found no indications of the presence of acid-potentiated inhibitory substances in the urine apart from D-glucaric acid. Notwithstanding these data definite proof for the enhanced

D-glucaric acid excretion during drug treatment requires isolation and identification. A more detailed study of the acid-potentiated inhibition in the urine of a drug-treated patient was made therefore. The aim was twofold:

1. Corroboration of the correctness of the enzymatic method of Marsh by showing the absence of acid-potentiated inhibitory substances in the urine other than D-glucaric acid.

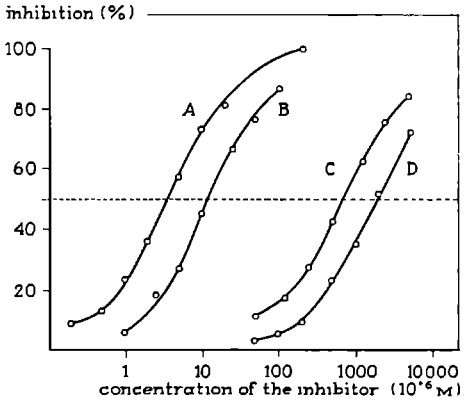

FIG. 11 *Inhibition of β-glucuronidase by various compounds.*

A, D-glucaro-(1→4)-lactone; B, D-glucaric acid boiled for 30 min at pH 3.5; C, D-galactaric acid boiled for 1 hr at pH 2; D, freshly prepared D-glucaric acid solution. From Levvy (1952).

2. Demonstration of the enhanced excretion of D-glucaric acid by isolation and identification.

An isolation procedure for D-glucaric acid has been given by Marsh (1963a). This procedure includes the removal of endogenous glucuronides by extraction with ethyl acetate, precipitation of the acid as a lead salt which subsequently is decomposed with H_2S, and isolation of the acid with phenylhydrazide from a solution treated with charcoal. Marsh started with 56 l urine containing 11.2 mg/l D-glucaric acid as estimated by the enzymatic assay and isolated 22 % of the total amount. Since the procedure of Marsh has a non-quantitative character and does not answer the question whether other substances which show an acid-potentiated inhibition, might be present in urine of drug-treated individuals, a different procedure for the isolation was followed in the present study.

The method of gradient elution from an ion-exchange column was applied,

followed by a further fractionation by paper chromatography. With this method which was described by Whiting and Coggins (1960) for sugar acids in fruits, a qualitative as well as a quantitative approach is possible. Finally part of the main fraction of the acid-potentiated inhibitor was treated with phenylhydrazide. The crystalline solid which deposited was weighed and identified as the bisphenylhydrazide of D-glucaric acid.

Isolation and identification

Urine was analyzed for acid-potentiated inhibition. This inhibition is expressed as an amount of 'apparent D-glucaric acid', meaning that the inhibitory action after acid potentiation would correspond with the presence of the indicated amount of D-glucaric acid if this substance is accepted to be the only cause of the inhibition. A 24-hr urine sample was collected from a patient chronically under treatment with phenobarbital and phenytoin (150 mg and 300 mg respectively every day). The amount of apparent D-glucaric acid was determined to be 89 mg. The urine was cooled, filtered and passed through a column of Dowex 50-WX4 (H^+form, 100/200 mesh, capacity 1250 meq). The effluent was evaporated under vacuum at 40° to a rest of about 200 g and further concentrated with freeze-dry technique to give a rest of 70 g with a total acid content of 210 meq. The material was extracted with ethyl acetate for

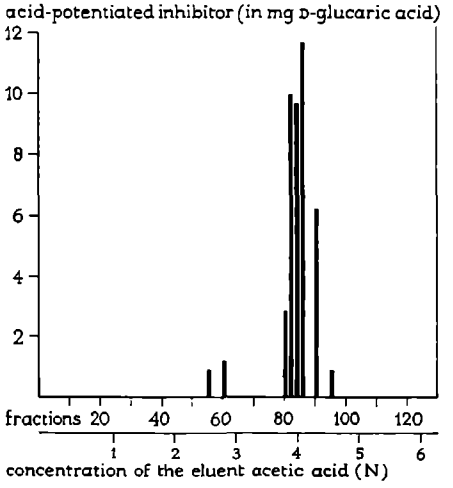

FIG. 12 *Elution of acid-potentiated inhibitors from a column of Dowex I-X2 (acetate form).*

The acid-potentiated inhibitors were obtained from urine of a patient under treatment with phenobarbital and phenytoin.

5 hours in a continuous liquid-liquid extractor at 25°. To reduce the content of strong acids the aqueous solution was passed through a column of Dowex I-X_2 (acetate form, 100/200 mesh, capacity 70 meq) and the effluent was again concentrated to give a rest with a total acid content of 76 meq. It was checked that no acid-potentiated inhibitor remained on the columns.

The gradient elution

As the following step in the analysis, the material was passed through a column (diameter 2.5 cm, height 40 cm) of Dowex I-X_2 (acetate form, 100/200 mesh, capacity 190 meq). The column was washed until the effluent obtained was neutral. The effluent displayed no inhibitory action. Then acetic acid solutions with concentrations increasing from 0 N to 6 N were successively passed through the column and 5 min-fractions with a volume of 50 ml each were collected. Every fifth fraction was checked on acid-potentiated inhibition. In the sequence of fractions acid-potentiated inhibition appeared in two area's (Fig. 12). The fractions 48 up to 65 were taken together and concentrated to a fraction A of approximately 3 ml. The same was done for the fractions 74 up to 99 yielding a fraction B. The amount of apparent D-glucaric acid in the fractions A and B was found to be 7.5 mg and 76.5 mg respectively.

Paper chromatography

The concentrates A and B obtained were studied further with descending chromatography on Whatman no. 1 paper, using the solvent system n-propanol: aq. ammonia 25 % (3:2 by vol.). The spots were developed with the aqueous acetone-silver nitrate method of Trevelyan et al. (1950) and fixed with 5 % sodium thiosulfate. Minute samples of the concentrates were chromatographed in two ways.

Method (a): The samples were chromatographed immediately after spotting them on the paper.

Method (b): The samples were brought on the paper with approximately 5 mm^3 2 N ammonia and chromatographed after a 24-hr stay in an atmosphere saturated with water and ammonia. In this manner possibly occurring lactones of D-glucaric acid are converted into the acid.

With both methods two chromatograms were made. One of each pair of chromatograms was developed with the silver nitrate reagent. The area's of the non-developed chromatogram of each pair corresponding with the developed spots were extracted with water and examined for acid-potentiated inhibition. D-glucaric acid and the (1→4)-lactone were used as references (Table 8). As demonstrated with method (a), the acid-potentiated inhibitors present in both concentrates A and B had R_F values of 0.22 and 0.30 respectively. If the

TABLE 8 *R_F values of compounds present in the concentrates A and B* obtained after gradient elution.*

Concentrate A		Concentrate B		D-glucaric acid	
				without (1→4)-lactone	with (1→4)-lactone
(a)	(b)	(a)	(b)	(b)	(a)
i 0.22	i 0.21	i 0.22	i 0.22	i 0.22	i 0.23
0.26	0.26				
i 0.30		i 0.30	0.30		i 0.30

* The concentrates were obtained from urine of a patient under treatment with phenobarbital and phenytoin.
Method (a): no pretreatment with 2 N ammonia.
Method (b): pretreatment with 2 N ammonia.
i: Compounds displaying acid-potentiated inhibition.
Solvent system n-propanol: aq. ammonia 25% (3:2 by vol.).

Note 1. Both untreated samples of the concentrates contain acid-potentiated inhibitors with the same R_F values as D-glucaric acid and the (1→4)-lactone.
2. After treatment with ammonia the samples of the concentrates A and B contain a non-inhibiting compound with R_F value 0.26 and 0.30 respectively.

inhibitory fractions with R_F value of 0.30 of concentrates A and B were extracted from the paper and chromatographed once more with method (b), an acid-potentiated inhibition was found at R_F value 0.22, whereas the acid-potentiated inhibition at R_F value 0.30 disappeared. This finding agrees with the supposition that the acid-potentiated inhibition at R_F value 0.30 is caused by a substance with a lactone character. It seemed highly probable that D-glucaric acid is involved in the acid-potentiated inhibition. If this supposition were true, it should be possible to isolate this substance as the bisphenylhydrazide derivative. However, the chromatograms showed that the concentrates A and B still contained impurities, so that further purification would have to precede a possible isolation. The concentrate A contained an important amount of an impurity with an R_F value not very different from that of the inhibitory compound itself. In connection with this complication and also with the fact that concentrate A contained only 7.5 mg 'apparent' D-glucaric acid, the inhibitory compound was not isolated from this concentrate.

Of concentrate B 55.7 % was used for the proposed experiment. In order to remove the non-inhibitory impurity (R_F 0.30), this fraction was chromatographed two times according to method (b) on 12 sheets (each 45 cm broad) of Whatman no. 1 paper. The area of every sheet displaying acid-potentiated in-

hibition was extracted with water. The combined extracts were evaporated to a dry residue.

Condensation with phenylhydrazide

The apparent amount of D-glucaric acid present in this residue was 38.5 mg. The dry residue was mixed with 2.5 ml water and centrifuged in order to remove paper fibres. The supernatant was heated with 0.4 g of phenylhydrazide hydrochloride and 0.1 g of anhydrous sodium acetate for 150 min at 100°. A yellowish crystalline solid deposited. After cooling overnight at 0° the crystallizate was sedimented by centrifuging, and washed with aq. 50 % (v/v) ethanol. The dry crystallizate, m.p. 208–210° (decomp.), had a weight of 48.1 mg. The supernatant was made alkaline with ammonia and extracted during 5 hours with ether in a liquid-liquid extractor in order to remove the phenylhydrazide. The apparent D-glucaric acid content of the ammoniacal solution was found to be 11.4 mg. Consequently 27.1 mg of apparent D-glucaric acid had disappeared from the solution[1].

The yellowish crystallizate was recrystallized two times from aq. 50 %(v/v) ethanol yielding 21.9 mg, m.p. 208–210° (decomp.) (Found: C,55.5; H,6.3; N,14.2%; Calc. for $C_{18}H_{22}N_4O_6$: C,55.4; H,5.7; N,14.4%). There was no depression of the melting point when the crystals were mixed with authentic D-glucaric acid bisphenylhydrazide, m.p. 209–210° (decomp.), and the infra-red spectra of the respective samples (KBr disk) could be superimposed. Consequently, it was accepted that the crystallizate was the bisphenylhydrazide of D-glucaric acid.

Conclusions

A 24-hr urine obtained from a patient treated with phenobarbital and phenytoin was studied. From this urine which contained 89.0 mg apparent D-glucaric acid, two fractions were obtained, a minor fraction and a main fraction. The amount of acid-potentiated inhibitor(s), present in these fractions, corresponded to 7.5 mg and 76.5 mg D-glucaric acid respectively. The inhibitor in both fractions showed, on application of the paper-chromatographic procedure, a behaviour which was identical with that of D-glucaric acid. For this reason it seemed probable that this inhibitor was D-glucaric acid.

From part (55.7 %) of the main fraction 48.1 mg of the bisphenylhydrazide of D-glucaric acid (corresponding to 26.0 mg D-glucaric acid) was isolated.

[1] The condensation of D-glucaric acid with phenylhydrazide will not proceed quantitatively. Under the conditions in question the degree of conversion of pure D-glucaric acid amounts to about 70%.

Consequently, it may be concluded that the 24-hr urine of the patient in question contained at least 46.7 mg D-glucaric acid. According to Marsh (1963a) the 24-hr urine of healthy humans contains about 10 mg 'apparent' D-glucaric acid. As reported in Table 7 we studied the 24-hr urine of 17 persons and found a mean amount of 6.5 mg $\pm$ 3.3 mg 'apparent' D-glucaric acid. This agrees rather well with the data of Marsh and justifies the conclusion that in the patient treated with phenobarbital and phenytoin the D-glucaric acid level was considerably enhanced. In the analytical procedure used no indications of the presence of acid-potentiated inhibitory substances, apart from D-glucaric acid, were obtained. It is concluded therefore that 'the apparent D-glucaric acid content' is identical with 'the D-glucaric acid content'.

4.4 Summary

In Chapter 2 some arguments were given for the supposition that the enhanced excretion of D-glucaric acid during drug treatment is based on the stimulation of the glucuronic acid pathway. The finding, reported by Marsh and Reid (1963) that during treatment of rats with barbital or chloretone an increased excretion of D-glucaric acid takes place, was the only experimental argument in this respect. In this chapter an extension of the experimental basis for the supposition mentioned is described. It was shown that in man as well as in the guinea pig (mammals unable to synthesize L-ascorbic acid) and the rat (mammal able to synthesize L-ascorbic acid) an increased excretion of D-glucaric acid takes place during drug treatment. For the guinea pig treated with barbital it was shown that this increase in the excretion is based on an increase in the biosynthesis of D-glucaric acid. It has been shown for rats that under such circumstances an increased level of L-ascorbic acid is present in the liver (Martin, 1961; Klinger and Koch, 1965a). This increased level is found to be due to an enhanced biosynthesis of L-ascorbic acid in the liver (Chapter 2). In the experiments described here it was shown that a strongly enhanced level of D-glucaric acid occurs in the liver of the rat treated with barbital. The fact that the enzyme involved in the final step of the D-glucaric acid formation occurs mainly in the liver and the fact that D-glucuronolactone is metabolized mainly in the liver, makes it probable that the enhanced D-glucaric acid level in the liver is due to biosynthesis in this organ. The parallels in the phenomena for the L-ascorbic acid and the D-glucaric acid excretion and production and the fact that both products have a common root in D-glucuronic acid, makes it highly probable that for D-glucaric acid like for L-ascorbic acid the enhanced formation after drug treatment is related to the stimulation of the glucuronic acid pathway under these circumstances. Now the question arose whether this

stimulation only takes place in the glucuronic acid pathway or also in the glucaric acid pathway. In the experiments described in this chapter it was made clear that the capacity of the final step in the D-glucaric acid synthesis is not enhanced during drug treatment. Concerning the lactonase(s) involved in the equilibrium between D-glucuronic acid and D-glucuronolactone no definite information is available. Taking into account the data concerning the L-ascorbic acid formation and the experiments described in this chapter, the assumption that the enhanced excretion and formation of D-glucaric acid is based on an enhanced formation of D-glucuronic acid during drug treatment is readily acceptable. This implies that it is not unreasonable to consider the enhanced excretion of D-glucaric acid in drug-treated animals as an indication of a stimulation of the glucuronic acid system.

The conclusions concerning the D-glucaric acid excretion based on the enzymatic method described by Marsh (1963a) must be considered as indirect conclusions. We could prove by isolation and chemical identification that during drug treatment (phenobarbital and phenytoin) enlarged quantities of D-glucaric acid are excreted in man. In the isolation procedure no indications were found of the presence in the urine of compounds interfering with the enzymatic assay of D-glucaric acid.

In the rat (see references in Table 1) and in the dog (Burns et al., 1957; Burns et al., 1963) drug treatment results in a stimulation of the glucuronic acid system as well as in an enhancement of drug metabolism in the liver. As was shown in this chapter, in guinea pig barbital treatment results in an enhanced formation and excretion of D-glucaric acid and in an enhancement of drug metabolism in the liver. Furthermore, it was found that drug treatment (aminophenazone, phenylbutazone, thiopental, phenobarbital-phenytoin) causes an increased D-glucaric acid excretion in man. On the basis of the analogy between the phenomena observed in guinea pig, rat and man, it may be assumed that in man a stimulation of the glucuronic acid pathway is the cause of the enhanced D-glucaric acid excretion. Taking into account that in man also during treatment with phenylbutazone or phenobarbital (Cheng et al., 1962; Burns et al., 1965) an enhanced metabolism of drugs takes place, it could be concluded that treatment with drugs results, as far as man, guinea pig, rat and dog are concerned, in a stimulation of the glucuronic acid system as well as in a stimulation of the hepatic enzyme systems involved in drug metabolism.

CHAPTER 5

A COMPARISON OF THE EFFECT OF DRUGS ON THE FORMATION AND EXCRETION OF L-ASCORBIC ACID AND D-GLUCARIC ACID AND A STUDY ON THE POSSIBLE RELATIONSHIP BETWEEN STIMULATION OF THE GLUCURONIC ACID SYSTEM AND A CHANGE IN GLUCURONIDATION UNDER THE INFLUENCE OF DRUGS

5.1 Introduction

As mentioned in the foregoing chapter, the enhanced D-glucaric acid excretion after drug treatment is not restricted to man or guinea pig but also takes place in mammals such as rats, which are able to synthesize L-ascorbic acid. A direct comparison of the influence of drugs on D-glucaric acid and L-ascorbic acid formation and excretion is as a matter of fact only possible in a study on animals which produce D-glucaric acid as well as L-ascorbic acid. The aim is to find out whether the changes in D-glucaric acid production and excretion follow the same pattern as those for L-ascorbic acid and D-glucuronic acid. In this respect also the question arises whether the increase in the excretion of D-glucaric acid in man and in mammals in general can be considered as a diagnostic sign of an enhanced activity in the glucuronic acid system and of an enhanced activity of the drug-metabolizing enzyme systems. Up to now in animal experiments and especially in the clinic the main emphasis was laid on the stimulation of drug metabolism by drugs (Ernster and Orrenius, 1965; Orrenius et al., 1965; Wilson and Fouts, 1966; Shuster and Jick, 1966a,b; Kato et al., 1966; Peraino et al., 1966; Alfred and Gelboin, 1967; Conney, 1967), while little or no attention was paid to the possible relationships between these phenomena and the changes in the glucuronic acid system.

5.2 The effects of barbiturates

5.2.1 The initial phase of stimulation of the glucuronic acid system in rats and dogs after treatment with barbital

It may be assumed that the stimulation of the glucuronic acid system as caused by drugs takes place in the liver cells. As a rule, however, the pheno-

menon is detected by measuring the enhanced urinary excretion of the products of the glucuronic acid system.

The quantity of L-ascorbic acid excreted per day under normal circumstances by the rat and expressed as a percentage of the total quantity synthesized per day is estimated as 15 percent (Conney et al., 1961) and 40 percent (Salomon and Stubbs, 1961). This indicates that under normal conditions there is a slight overproduction of L-ascorbic acid. During treatment with stimulating drugs there is a clear overproduction. This overproduction manifests itself not only in an increase of the L-ascorbic acid content in the urine but also in that of the tissues. Conney et al. (1961) showed that under the influence of strong stimulating drugs such as barbital and pentobarbital, the quantity of L-ascorbic acid in the animal tissues is about doubled. From the literature it can be concluded that under the influence of stimulating drugs the increase in the L-ascorbic acid level differs for the various tissues. In this respect the question arises in which tissues or biological fluids (urine, blood) the rise in the L-ascorbic acid content as a result of stimulation can be detected soonest.

Martin (1961) reported that during treatment of rats with chloretone the L-ascorbic acid level in the liver increased with 50 percent, in the muscular tissue with 100 percent and in the urine to a manifold of the original concentration. He also observed that the level in the blood hardly changed. Klinger and Koch (1965a) observed in the rat that after treatment with barbital the maximum value of the L-ascorbic acid content in the liver, which is about 140 percent of the normal value, is reached after 5 hours. During the period of the enhanced L-ascorbic acid level in the liver no changes in the levels of blood, kidney and brain tissue were observed. Treatment with barbital resulted in an increase of the L-ascorbic acid level in the adrenals (Klinger and Koch, 1965b).

TABLE 9 *Effect of barbital treatment* on the amount of L-ascorbic acid in rat liver.*

Time after injection hr	L-ascorbic acid µmoles/g liver		P value
	control	treated	
2	0.93 ± 0.10 (6)	0.99 ± 0.06 (6)	>0.10
3	0.89 ± 0.06 (6)	1.03 ± 0.08 (6)	<0.01

*Male rats, weighing 180–200 g, were given injections of sodium barbital 150 mg/kg i.p. at zero time. The control animals were given saline.
P values were obtained by applying the two-sided Wilcoxon two-sample test. Numbers in parenthesis indicate number of animals on which mean is based. Means with S.D. are presented.

From the experiments of Klinger and Koch (1965a) it can be seen that already 3 hours after i.p. application of barbital to rats the L-ascorbic acid level in the liver is increased. The activity of the drug-metabolizing enzyme systems in the liver however, is not yet found to be increased 3 hours after i.p. application of phenobarbital (Ernstei and Orrenius, 1965), which indicates that for this phenomenon there is a longer latency time than for the response in the L-ascorbic acid levels. The question how long the latency time for the enhanced L-ascorbic acid and D-glucaric acid formation and for the enhanced drug metabolism is, is of importance in relation to the question whether these phenomena are based on *de novo* synthesis of enzymes. For that reason further experiments

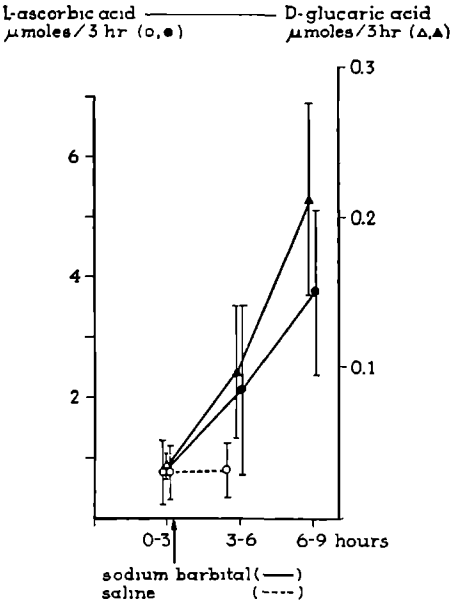

FIG. 13 *Effect of barbital on the urinary excretion of L-ascorbic acid and D-glucaric acid in rats.*

Male rats, weighing 45–60 g, were used. After collecting urine over a 3-hr period a group of 8 rats was injected with sodium barbital 140 mg/kg i.p. (see arrow). Urine was collected in two consecutive 3-hr periods. L-ascorbic acid and D-glucaric acid excretions were determined in the three periods. After collecting urine over a 3-hr period a control group of 6 rats was injected with saline i.p. and urine was collected over another 3-hr period. L-ascorbic acid excretion was determined in the two periods. Means with S.D. are presented. The 3-hr intervals after treatment were compared with the 3-hr interval before treatment. Significant differences are represented by closed symbols ($P \leq 0.05$; two-sided Wilcoxon signed-rank test).

were designed. From these experiments it was found that 2 hours after drug treatment there was no clear indication of an increase in the L-ascorbic acid content in the liver, while after 3 hours the increase was significant (Table 9). The excretion in the urine of L-ascorbic acid and D-glucaric acid in the 3-hr period following the i.p. application of barbital increased significantly (Fig. 13).

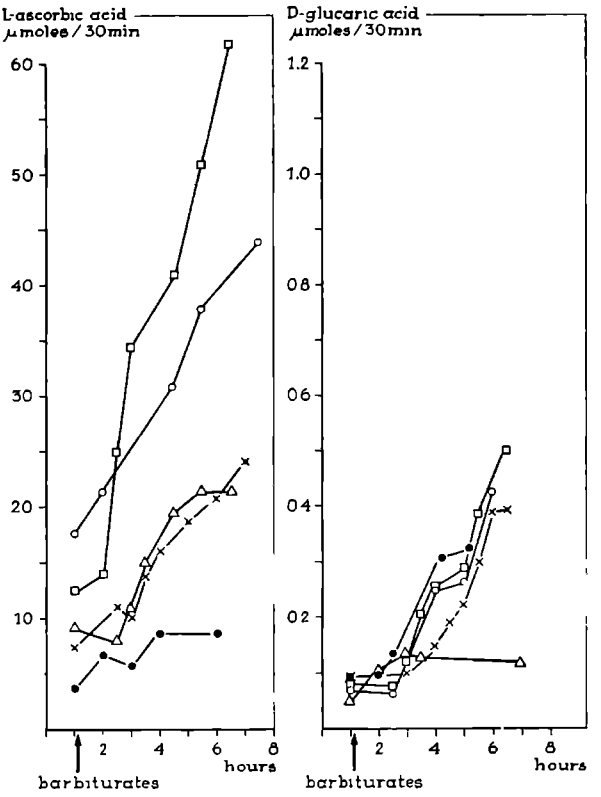

FIG. 14 *Effect of barbiturates (barbital and thiopental) on the urinary excretion of L-ascorbic acid and D-glucaric acid in dogs.*

Five male dogs, weighing 18–24 kg, fasted 12 hr. The dogs were catheterized at zero time, and at 1 hr control urine was collected. After that the dogs were anaesthetized with sodium barbital 175 mg/kg and sodium thiopental 25 mg/kg i.v. The dogs were supplemented with 2–5 mg/kg per hr sodium thiopental whenever necessary. If possible, urine was collected at 2 hr by catheterization. After that urine was collected from the cannulated ureters at 30-min periods. Each point in the figure corresponds with the urine of an interval that finishes at the indicated time. Each symbol represents one dog. The values given at 1 hr and 2 hr are obtained by dividing the amount of L-ascorbic acid and D-glucaric acid in the 1-hr fractions by 2.

These results indicate that under the influence of barbital the latency time for the stimulation of the glucuronic acid system is shorter than 3 hours, but probably longer than 2 hours. One should be aware of the fact that in order to find the lag time between the initiation of the stimulation mechanism and the manifestation of the effect, the time required for the absorption of the drug should be deducted from the latency time.

In order to become informed on the changes during the 2-hr period after drug application, it is necessary to use animals from which practically continuously quantities of urine, large enough to be analyzed on their L-ascorbic acid and D-glucaric acid content, can be obtained. For this reason experiments were performed on dogs in which the ureters were cannulated. As stimulating drugs a combination of barbital and thiopental was used and applied intravenously (i.v.) in order to reduce the time required for resorption and distribution of the drug to a minimum. Moreover, the drugs used served as anaesthetics for the animals. No premedication was used. Under these experimental conditions the first measurements could be made about 1 hour after the drug application (Fig. 14). From the results presented in this figure it can be seen that it takes about 2 hours before a clear increase in the excretion is observed. As known from biochemistry the time required for *de novo* synthesis of hepatic enzymes after oral application of various stimuli, such as hormones and substrates, can be as short as 2 hours (Steele, 1966). This implies that the latency time of about 2 hours observed for the enhanced formation and excretion of the products of the glucuronic acid system in the liver do not exclude the possibility that *de novo* synthesis of enzymes might be involved. The question about this *de novo* synthesis of enzymes is dealt with in Chapter 6.

5.2.2 The influence of the dosis of barbital used on the excretion of some products of the glucuronic acid system and the time pattern of this excretion in rats

It is of interest to know, in order to cause enhanced excretions of the various products of the glucuronic acid system such as L-ascorbic acid, D-glucaric acid and D-glucuronic acid, whether the same doses of stimulating drug are needed and whether the time courses of the excretions are similar or different. For this purpose we have studied the dose-effect relationship for barbital on rats, comparing in one set of experiments the excretion of L-ascorbic acid and D-glucuronic acid (Fig. 15) and in the second set of experiments the excretion of L-ascorbic acid and D-glucaric acid (Fig. 16). The lowest dose of barbital (20 mg/kg) used resulted already in an increased excretion of L-ascorbic acid. An enhanced excretion of D-glucaric acid was observed after a dose of 60 mg/kg (20 mg/kg on the first and 40 mg/kg on the second day). An enhancement of

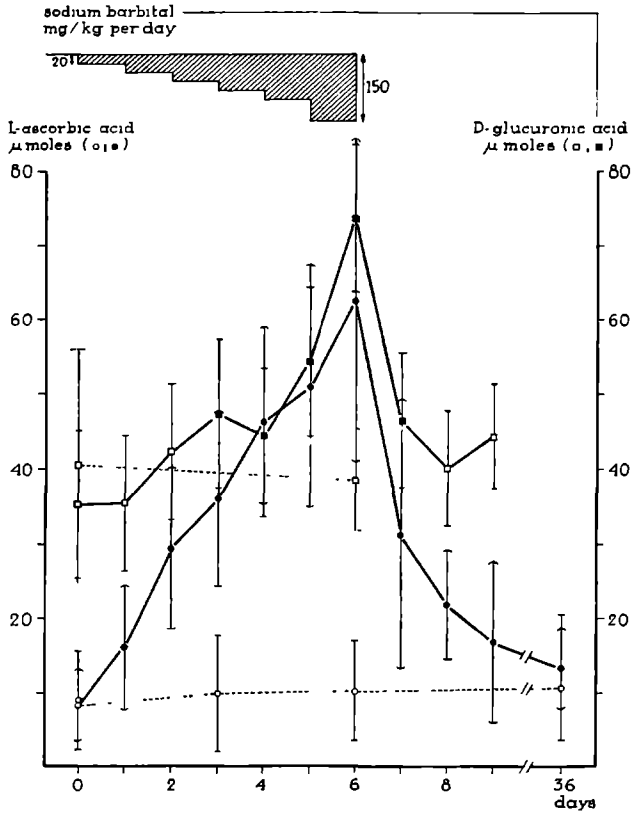

FIG. 15 *Effect of cumulative doses of barbital on the urinary excretion of L-ascorbic acid and D-glucuronic acid in rats.*

Female rats, weighing 180–220 g, were used. A group of 8 animals was injected i.p. with saline on day zero and with sodium barbital as indicated in the figure on the following days. The quantity administered per day was given in a single dose. A group of 8 control animals was injected with saline every day. 24-hr Urine was collected and used for the assay of the L-ascorbic acid and D-glucuronic acid content. Means with S.D. are presented. Excretions during and after treatment are compared with excretion before treatment (day zero). Significant differences are represented by closed symbols ($P \leq 0.05$; two-sided Wilcoxon signed-rank test).

Note After administration of 20 mg/kg and 40 mg/kg sodium barbital at day 1 and 2 respectively, the increase in the L-ascorbic acid excretion is significant, while the increase in the D-glucuronic acid excretion is not. After finishing the drug treatment a similar pattern is observed.

the D-glucuronic acid excretion was observed only after a dose of 120 mg/kg (20, 40 and 60 mg/kg successively). The first day after stopping the application of the drug there is a steep drop in the levels of all 3 products measured, while the level of the D-glucuronic acid excretion returns to normal already on the second day after stopping the application. On the 11th day after stopping the application of barbital, the L-ascorbic acid as well as the D-glucaric acid excretion appeared to be still slightly increased. For L-ascorbic acid, measurements were made for a still longer period and it was found that even on the

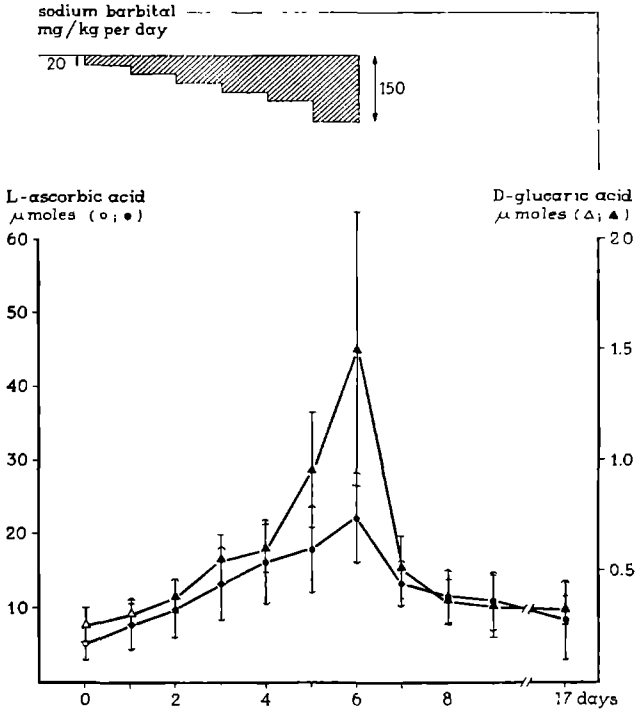

FIG. 16 *Effect of cumulative doses of barbital on the urinary excretion of L-ascorbic acid and D-glucaric acid in rats.*

Female rats, weighing 180–220 g, were used. A group of 7 animals was injected i.p. with saline on day zero and with sodium barbital as indicated in the figure on the following days. The quantity administered per day was given in a single dose. 24-hr Urine was collected and used for the assay of the L-ascorbic acid and D-glucaric acid content. Means with S.D. are presented. Excretions during and after treatment are compared with excretion before treatment (day zero). Significant differences are represented by closed symbols ($P \leq 0.05$; two-sided Wilcoxon signed-rank test).

Note The increase in the L-ascorbic acid and D-glucaric acid excretion after administration of 20 mg/kg and 40 mg/kg sodium barbital at day 1 and 2 respectively is significant. Note also the significant response after finishing drug treatment.

30th day after stopping the application this level still did not reach the original level. The enhanced excretion of D-glucuronic acid after barbital treatment is difficult to interpret because of the high D-glucuronic acid content in the urine before treatment. It is probable that a considerable part of this content originates from the food ingested by the animals. It is possible that after the barbital treatment a response in the D-glucuronic excretion occurs which is of the same character as that of the L-ascorbic acid excretion, but that this response is obscured by the high base level of the D-glucuronic acid excretion. The results are in agreement with the suggestion made in Section 4.1 that for the detection of an enhanced activity in the glucuronic acid system the excretion of L-ascorbic acid and D-glucaric acid is a more sensitive parameter than that of D-glucuronic acid.

What is the cause of the persistent response in the L-ascorbic acid and D-glucaric acid excretion after cessation of the drug treatment? A gradual disposal by the animal of the L-ascorbic acid accumulated in the tissues, may be one of the factors. A second possibility is the persistence for some time of the enhanced formation of L-ascorbic acid and also of D-glucaric acid due to the enlargement of the liver, which is observed during and after drug treatment. Barbital, phenobarbital and many other drugs cause a hypertrophy of the liver which gradually disappears after cessation of the drug treatment, so that after about 14 days, as was described by Kunz et al. (1966a), the original size of the organ is reached again. This implies that for the first two weeks after cessation of the drug administration the hypertrophy of the liver may be the factor in the persistent enhanced excretion of L-ascorbic acid and D-glucaric acid. The enhanced L-ascorbic acid excretion, however, is still evident 4 weeks after the last dose, and therefore cannot be explained by hypertrophy alone. Another aspect is the possibility of an enhanced synthesis of enzymes as caused by barbital. This would imply that after cessation of drug treatment, depending on the half-life time of these enzymes, a certain persistence in the enhanced formation and attendant excretion of L-ascorbic acid and D-glucaric acid might be expected. From experiments to be described in Section 6.1.2 it is concluded however, that in the stimulation of the glucuronic acid system by barbital, *de novo* synthesis of enzymes does not play a predominant role. Hollmann and Neubaur (1967) found indications that the half-life time of the enzymes UDPG dehydrogenase and UDP glucuronyltransferase which are involved in the glucuronic acid system, is only a matter of hours. This implies that a lasting enhanced level of the enzymes mentioned is not an essential factor for the persistence of the enhanced excretions of L-ascorbic acid and D-glucaric acid. A main factor may be found in the fact that the animals can only slowly dispose of the drug used. Frey et al. (1959) reported that in a man's urine even 27 days

after application of barbital, the drug can still be detected. Barbital is very slowly metabolized and eliminated. This holds for man as well as for the rat (Ebert et al., 1965). After application of 3-methylcholanthrene to the rat an enhanced excretion of L-ascorbic acid during some months is reported (Dayton et al., 1964). It is known that this compound too persists for a very long time in the animal. The conclusion may be that the persistence of the drug in the body is the cause of the continuous L-ascorbic acid response occurring weeks after the last application of the drug.

5.2.2.1 Differences in individual levels of L-ascorbic acid excretion in rats under standard conditions and during barbital treatment

In the course of the study it appeared that the individual differences in the urinary L-ascorbic acid excretion in untreated rats of equal sex and strain were greater than those in the D-glucaric acid excretion. As may be seen in Table 10 (controls) this fact finds expression in the relatively large standard deviations (S.D.'s) of the L-ascorbic acid excretion as compared with the S.D.'s of the D-glucaric acid excretion. It appeared that, although the amount of L-ascorbic acid excreted in the urine strongly varies between the experimental animals, the daily excretion per individual is fairly constant (Fig. 17). Moreover, a positive correlation was found between the increase in L-ascorbic acid excretion during barbital treatment and the excretion level before treatment.

According to Chatterjee et al. (1961) the *in vitro* activity of the hepatic L-gulonolactone oxidase, involved in the terminal step of the L-ascorbic acid synthesis in rats of the same strain differs greatly from animal to animal. It is likely that the same holds for the *in vivo* capacity to synthesize L-ascorbic acid and that this finds its expression in the great differences in the urinary excretion levels.

5.3 The influence of a variety of drugs on the excretion of the products of the glucuronic acid system in rats

5.3.1 Experiments

For a variety of drugs and compounds, an enhancement of the L-ascorbic acid excretion in the rat is reported in the literature (Table 1). As far as the D-glucaric acid excretion and the D-glucuronic acid excretion are concerned information is restricted to barbital, chloretone and 3-methylcholanthrene. Although these compounds are known to cause an enhancement of L-ascorbic acid biosynthesis and of drug metabolism, only the first two compounds men-

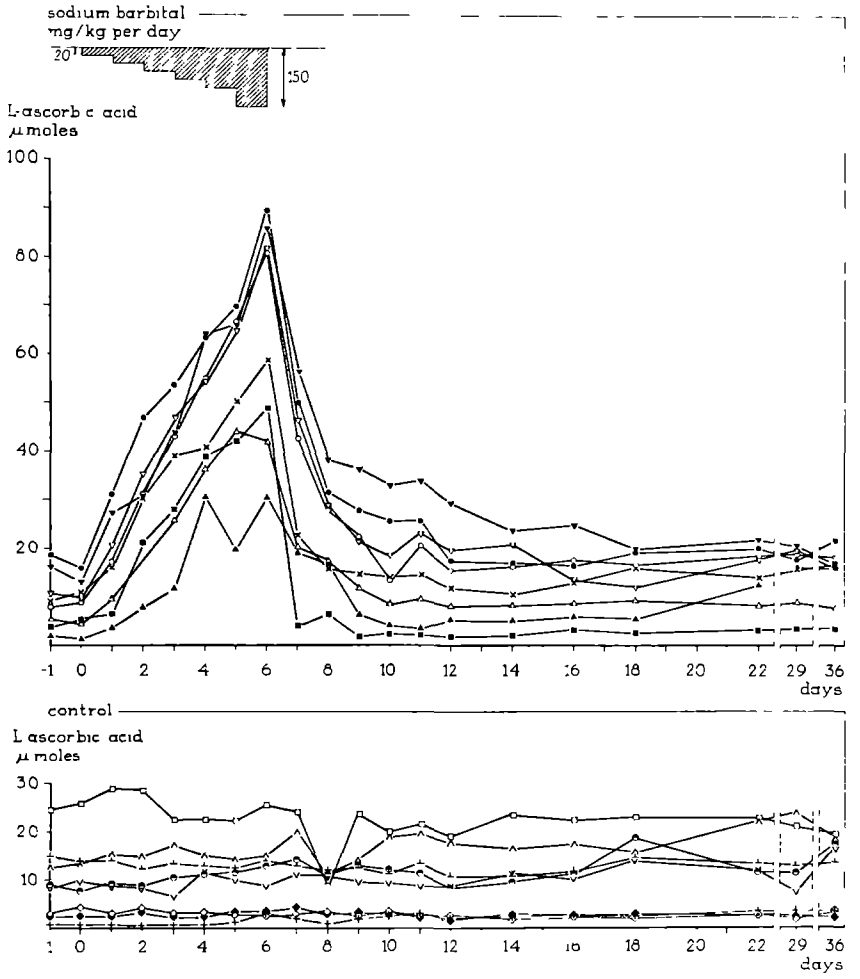

FIG. 17 *Differences in individual levels of L-ascorbic acid excretion in rats under standard conditions (lower part of the figure) and during barbital treatment.*

The individual levels of the L-ascorbic acid excretion in the rats described in Fig. 15 are given.

Note the constant amount of L-ascorbic acid excreted every day by the control animals.

Note also the greater response of the treated animals which have a high base level before treatment. There is a positive correlation between the increase in L-ascorbic acid excretion after administration of sodium barbital 150 mg/kg (24-hr excretion after administration of sodium barbital minus 24-hr excretion at day zero) and the base level before treatment (Kendall's rank correlation test).

TABLE 10 *Urinary excretion of L-ascorbic acid, D-glucaric acid and D-glucuronic acid in rats on the first day of drug treatment.*

Treatment*	L-ascorbic acid** µmoles/24 hr			D-glucaric acid** µmoles/24 hr			D-glucuronic acid** µmoles/24 hr		
	control	treated	P value	control	treated	P value	control	treated	P value
Aminophenazone, 125 mg/kg orally	7.0 ± 2.1 (6)	14.8 ± 2.9 (6)	0.03	0.33 ± 0.08	0.38 ± 0.06	>0.10			
Borneol, 400 mg/kg orally	3.0 ± 1.7 (7)	7.2 ± 2.0 (7)	<0.01	0.37 ± 0.10	0.33 ± 0.13	>0.25			
Nikethamide, 125 mg/kg orally	14.3 ± 6.9 (6)	55.6 ± 18.6 (6)	<0.01	0.34 ± 0.09	0.41 ± 0.12	>0.10	36.6 ± 10.8	24.1 ± 9.3	0.05
Phenylbutazone, 125 mg/kg orally	2.7 ± 0.6 (12)	14.8 ± 7.7 (8)	<0.01	0.31 ± 0.04	0.36 ± 0.09	0.10			
Sodium barbital, 150 mg/kg i.p.	12.8 ± 8.4 (6)	32.3 ± 18.4 (6)	0.03	0.42 ± 0.11	2.1 ± 0.6	0.01	24.5 ± 7.4	35.3 ± 7.9	0.01
Sodium thiopental, 40 mg/kg i.p.	6.3 ± 5.3 (7)	15.2 ± 10.3 (7)	0.05	0.42 ± 0.09	0.35 ± 0.08	>0.10			
Tolbutamide, 125 mg/kg orally	6.9 ± 1.9 (6)	8.0 ± 2.2 (6)	0.03	0.22 ± 0.04	0.21 ± 0.05	>0.25			
DDT, 30 mg/kg orally	14.3 ± 6.9 (6)	67.0 ± 23.0 (6)	<0.01	0.34 ± 0.09	0.35 ± 0.12	>0.25	36.6 ± 10.8	33.9 ± 6.8	>0.25
D-glucuronolactone, 4000 mg/kg orally (about 4500 µmoles per rat)	6.2 ± 1.1 (8)	19.6 ± 4.8 (8)	<0.01	0.39 ± 0.08	12.2 ± 5.6	<0.01			

* Female rats, weighing 180–220 g, were used. Aminophenazone, borneol, sodium barbital, tolbutamide and DDT were given in single doses; sodium thiopental, nikethamide and phenylbutazone were given in 2 doses: at zero time and at 8 hr. D-glucuronolactone was given in 4 doses: at zero time, at 4 hr, 8 hr and 12 hr.

** Collection of urine was started after the first dose.

The figures and P values are calculated as indicated in Table 9.

tioned were effective, and 3-methylcholanthrene was ineffective with respect to the enhancement of the D-glucaric acid excretion (Marsh and Reid, 1963). With respect to the D-glucuronic acid excretion only barbital was very active. In order to extend the information on the influence of several drugs on the excretion of D-glucaric acid, L-ascorbic acid and D-glucuronic acid one or two doses of the drug were given to rats within a 24-hr period. For purposes of comparison the effect of D-glucuronolactone was also studied. D-glucuronolactone is a precursor of L-ascorbic acid and D-glucaric acid (Sections 1.1.2 and 1.1.3). The 24-hr portions of urine produced after the first application of the drugs were analyzed; the D-glucuronic acid content of the urine was studied only as far as those drugs are concerned which themselves are not or only slightly involved in the formation of glucuronides. The results are presented in Tables 10 and 11.

TABLE 11 *Urinary excretion of L-ascorbic acid and D-glucaric acid in rats* treated with low doses of D-glucuronolactone*

Day	D-glucuronolactone administered** daily dose μmoles per rat	Urinary excretion μmoles/24 hr L-ascorbic acid	P value	D-glucaric acid	P value
1	0	6.5 ± 2.4		0.18 ± 0.06	
2	12.5	6.2 ± 2.5	>0.25	0.22 ± 0.09	>0.25
3	25	7.8 ± 2.9	0.03	0.34 ± 0.04	0.02
4	50	8.4 ± 2.9	0.03	0.46 ± 0.10	0.02
5	225	8.4 ± 3.3	0.08	0.82 ± 0.20	0.03

* 7 Female rats, weighing 180–220 g, were used.
** D-glucuronolactone was given orally in 4 doses: at zero time, at 4 hr, 8 hr and 12 hr. After collecting a 24-hr urine on the 1st day, the rats were given D-glucuronolactone on the following days.
Means with S.D. are shown. The 24-hr intervals after treatment were compared with the 24-hr interval before treatment (two-sided Wilcoxon signed-rank test).

From Table 10 it can be seen that with the exception of barbital, no increase in the D-glucaric acid and D-glucuronic acid excretion is observed, although the L-ascorbic acid excretion is increased for all drugs. During nikethamide treatment the excretion of D-glucuronic acid is even lowered. The conclusion might be that neither the excretion of D-glucuronic acid nor the excretion of D-glucaric acid are suitable as an indicator of an enhanced activity in the glucuronic acid system. The L-ascorbic acid excretions both after drug treatment and after

loading with high doses of D-glucuronolactone are of the same order. This does not apply to the excretion of D-glucaric acid. After treatment with drugs, in no case is such a high D-glucaric acid excretion reached as after D-glucuronolactone loading. After administration of low doses of D-glucuronolactone the D-glucaric acid excretion is relatively more pronounced than the L-ascorbic acid excretion (Table 11). The differences in L-ascorbic acid and D-glucaric acid excretion obtained with exogenous D-glucuronolactone as compared to the situation in drug-treated and in untreated animals will be dealt with more extensively in the discussion (Section 5.3.2).

One should be well aware of the fact that the data of Table 10 are obtained with rats, animals which apparently easily synthesize L-ascorbic acid. It is possible that the lack of an enhanced formation and excretion of D-glucaric acid and D-glucuronic acid has to be ascribed to the ease with which the intermediates of the glucuronic acid pathway find their way along the ascorbic acid pathway and the xylulose pathway. The experiments of Marsh (1963a) and Okada et al. (1964) indicate that D-glucuronolactone applied to the rat results in a relatively slight formation of D-glucaric acid, if compared with species that cannot synthesize L-ascorbic acid. These results could be confirmed by us with respect to rats and guinea pigs (compare Table 5 with Table 10). If endogenous D-glucuronic acid synthesized to a high extent during drug treatment, is processed further according to the same species-dependent pattern of D-glucuronolactone, it may be expected that men and guinea pigs give a stronger D-glucaric acid response upon drug treatment than rats. The investigation of some of the drugs found to be ineffective with respect to the D-glucaric acid response in rats would have to be made in e.g. guinea pigs in order to

TABLE 12 *Urinary excretion of D-glucaric acid in guinea pigs* under treatment with aminophenazone or thiopental.*

Treatment	D-glucaric acid µmoles/24 hr		
	control	treated	P value
Aminophenazone, 125 mg/kg orally	0.37 ± 0.11 (8)	0.49 ± 0.18 (6)	>0.25
Sodium thiopental, 40 mg/kg i.p.	0.29 ± 0.12 (6)	0.41 ± 0.24 (6)	>0.10

*Female guinea pigs, weighing 300–400 g, were used.
Conditions of this experiment were similar to those of Table 10.
Collection of urine was started after the first injection and after the oral dose.
The figures and P values are calculated as indicated in Table 9.

get more information on this point. Table 12 summarizes the results obtained. In the guinea pig no significant increase in the D-glucaric acid excretion is obtained in the 24-hr urine collected after application of high doses of aminophenazone or thiopental. To a certain degree this is unexpected. A plausible explanation is that the endogenous D-glucuronic acid is metabolized primarily via the xylulose pathway in mammals such as the guinea pig and man, species that cannot synthesize L-ascorbic acid.

In man an enhanced D-glucaric acid excretion is observed after application of aminophenazone and thiopental, however, in these experiments the drugs concerned have been applied for ten days or longer (Table 7). From the foregoing it appears clearly that animal experiments have to be performed in which a more chronic treatment with the stimulating drugs is applied.

In the next series of experiments rats were treated for three days with barbital, aminophenazone, nikethamide or phenylbutazone. The excretions were studied in 24-hr urine which in the case of D-glucaric acid was pooled (Fig. 18). These experiments make clear that under these circumstances barbital again is very effective as a stimulator for the D-glucaric acid excretion, while as far as

TABLE 13 *Urinary excretion of D-glucaric acid in rats on the fourth day of treatment with drug, and liver weight of rats after the fourth day of treatment.*

Treatment*	D-glucaric acid** µmoles/24 hr			Liver weight***		
	control	treated	P value	control	treated	P value
Aminophenazone, 125 mg/kg	0.31 ± 0.07 (6)	0.51 ± 0.16 (6)	0.03	5.0 ± 0.5	5.9 ± 0.7	0.03
Nikethamide, 125 mg/kg	0.26 ± 0.15 (6)	0.98 ± 0.33 (6)	<0.01	4.7 ± 0.4	5.0 ± 0.2	0.10
Phenylbutazone, 125 mg/kg	0.31 ± 0.04 (6)	0.46 ± 0.04 (6)	<0.01	4.7 ± 0.3	6.0 ± 0.8	<0.01
Borneol, 400 mg/kg	0.20 ± 0.07 (7)	0.69 ± 0.39 (8)	0.01	4.7 ± 0.5	5.3 ± 0.5	0.06
Salicylic acid, 400 mg/kg	0.26 ± 0.15 (6)	0.35 ± 0.21 (7)	>0.25	4.7 ± 0.4	5.5 ± 0.4	<0.01
Sodium thiopental, 40 mg/kg	0.20 ± 0.07 (7)	0.35 ± 0.17 (7)	0.07	4.7 ± 0.5	5.5 ± 0.3	<0.01
DDT 30 mg/kg	0.28 ± 0.04 (6)	0.28 ± 0.07 (6)	>0.25	4.7 ± 0.3	5.3 ± 0.4	0.01

* Female rats, weighing 180–220 g, were used. The drugs were given orally in 2 doses at zero time and 8 hr.

** Collection of urine was started at 72 hr.

*** Liver weight was determined at 96 hr.

The figures and P values are calculated as indicated in Table 9.

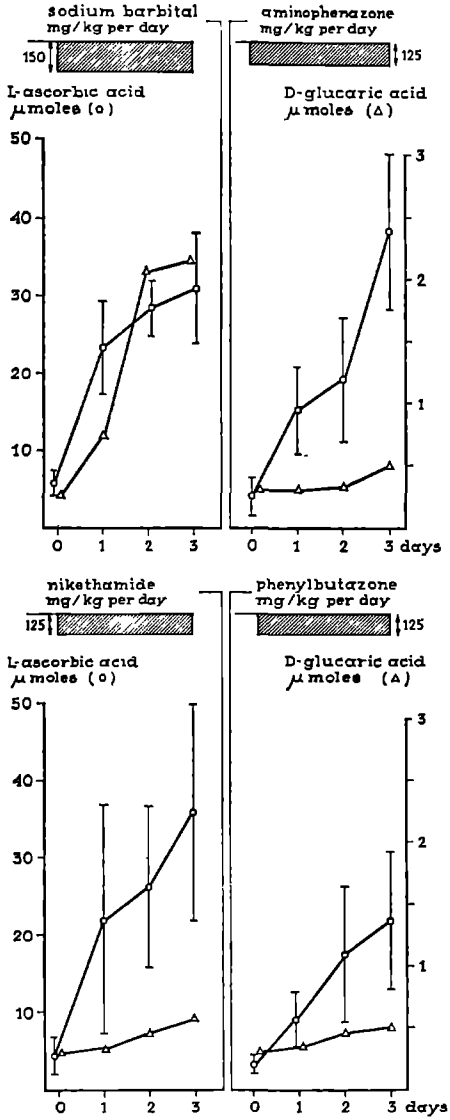

FIG. 18 *Effect of repeated administration of several drugs on the excretion of L-ascorbic acid and D-glucaric acid in rats.*

Groups of 5 female rats, weighing 180–200 g, were treated orally. Urine was collected in 24-hr periods. At day zero control urine was collected. As far as L-ascorbic acid is concerned means with S.D. are presented; as far as D-glucaric acid is concerned means determined in the pooled urine are presented.

the other drugs are concerned there is a slight indication of an enhanced D-glucaric acid excretion. All the drugs have a strong influence on the L-ascorbic acid excretion. In order to get more definite information another series of experiments was performed with rats treated for 4 days with aminophenazone, nikethamide, phenylbutazone, salicylic acid, DDT, thiopental and borneol. In this case the individual 24-hr urine portions were analyzed. The results summarized in Table 13 show that a significant increase of the D-glucaric acid excretion is observed only after treatment with aminophenazone, nikethamide, phenylbutazone and borneol. However, with the exception of salicylic acid, all the drugs used are known to be effective stimulators of the L-ascorbic acid excretion in the rat.

An important aspect to be considered after more chronic drug treatment in relation to the enhanced formation of products of the glucuronic acid system is liver growth. As said before (Section 5.2.2), after drug treatment an increase in the weight of the liver is observed. This enlargement of the liver is mainly due to a hypertrophy of the liver cells (Kunz et al., 1966a). Increase of the number of cells (hyperplasia) has only been observed after prolonged treatment (3–6 months) with certain of the halogen-alkyl anaesthetics. As a matter of fact the enlargement of the quantity of liver tissue as such might be the cause of the increase in the production of L-ascorbic acid and D-glucaric acid and therefore of the increase of the excretion of these products after chronic treatment with the drugs. However, nikethamide and also carcinogenic hydrocarbons do not cause liver enlargement (Kirchberg, 1966). In our own experiments the drug treatment resulted in several cases in an enlargement of the weight of the liver. The experimental results summarized in Table 13 show that in rats pretreated with nikethamide or borneol no significant increase in the weight of the liver is observed, while there is nevertheless a clear increase in the excretion of D-glucaric acid. In rats treated with DDT or salicylic acid, on the other hand, there is a significant increase of the liver weight, while an enhanced excretion of D-glucaric acid is not observed. So there is no direct relation between the increase in the liver weight and the enhancement of the D-glucaric acid excretion during drug treatment. The increase of the weight of the liver as such therefore is not the main cause of the increase in the excretion of D-glucaric acid. It is assumed that the differences in the response to the various drugs as far as the excretion of D-glucaric acid is concerned are due to differences in the effect of these drugs on the glucuronic acid system (Section 5.3.2).

5.3.2 Discussion

In radio-active tracer studies Evans et al. (1960) and Conney et al. (1961)

showed that treatment of rats with barbital, chloretone and 3-methylcholanthrene brings about an enhanced conversion of D-hexose into the products of the glucuronic acid system such as D-glucuronic acid, L-gulonic acid and L-ascorbic acid (Section 2.3.1). A closer analysis of their data shows that there is a difference between the response during treatment with barbital and the response during treatment with chloretone and 3-methylcholanthrene. During treatment with barbital there is an increase in the urinary excretion of labeled L-ascorbic acid as well as labeled D-glucuronic acid, while during treatment with chloretone and 3-methylcholanthrene a similar increase in the L-ascorbic acid excretion but little increase in the excretion of labeled D-glucuronic acid takes place. Dayton et al. (1965) also report that during treatment of rats with chloretone or with 3-methylcholanthrene little increase in the excretion of D-glucuronic acid occurs, while during treatment with barbital a clear increase takes place. No biochemical interpretation of these differences in the responses could be given up to now (Dayton et al., 1965; Marsh and Reid, 1963).

Differences in the excretion of D-glucuronic acid and L-ascorbic acid during treatment with various drugs were also mentioned in relation to the experiments described in Section 5.3.1. These experiments confirm the results of Burns et al. (1957), Nitze and Remmer (1962) and Dutton et al. (1965) in which was shown that in animals treated with barbital the D-glucuronic acid excretion is enhanced. The observation of Dutton et al. (1965) that not all drugs stimulating drug metabolism, e.g. chloretone and 3-methylcholanthrene, cause a strong increase in the D-glucuronic acid excretion, is extended by the results of our own experiments, in which it was found that during treatment of rats with nikethamide and DDT the excretion of D-glucuronic acid was not increased, while the excretion of L-ascorbic acid was (Table 10).

After drug treatment the response in the L-xylulose excretion in pentosuriacs resembles that of L-ascorbic acid in the rat. Within 24 hours after administration of the first dose of borneol or aminophenazone both the excretion of L-xylulose in the pentosuriac and the L-ascorbic acid excretion in the rat are enhanced.

As far as the excretion of D-glucaric acid is concerned, it can be concluded that the enhancement after treatment with barbital is much greater than that observed with the other drugs investigated (Table 10; Fig. 18). In the experiments of Marsh and Reid (1963) an indication is found for an increase in the D-glucaric acid excretion during barbital and chloretone treatment and little or no increase during 3-methylcholanthrene treatment. In general it can be said that with low doses of the drug (Figs. 15, 16) or application of short duration (Table 10; Fig. 18) the response in the L-ascorbic acid excretion is much clearer than the response in the D-glucaric acid and D-glucuronic acid excretion. With

high doses of the drugs after prolonged treatment (4 days) responses are observed in the L-ascorbic acid as well as in the D-glucaric acid excretion, while the D-glucuronic acid excretion is not studied in detail under these circumstances (Figs. 15; Table 13). These differences in the responses as far as the stimulation of the glucuronic acid system by various drugs is concerned are not in accordance with the supposition that the enhanced excretion of D-glucuronic acid, D-glucaric acid and L-ascorbic acid are related in a direct and uncomplicated way to an enhanced formation of D-glucuronic acid in the liver.

In efforts to interpret the findings from the literature and those obtained in the experimental part of this chapter, the following points should be taken into consideration. Under normal conditions the D-glucaric acid levels in the liver and in the urine of the rat are very low. The content in the liver is about a 200-fold smaller than the L-ascorbic acid content (Compare Table 3 with Table 9; the L-ascorbic acid contents in the liver of males and females are of the same order: Koch and Klinger, 1963). The quantity of D-glucaric acid excreted in urine is about a 10- to 40-fold lower than that of L-ascorbic acid (Table 10). After application of high doses of D-glucuronolactone to the rat and the guinea pig the D-glucaric acid excretion increases about 30-fold and more than a 200-fold respectively (Tables 5 and 10), while the L-ascorbic acid excretion in the rat is only increased about 3-fold. Also after low doses of D-glucuronolactone the D-glucaric acid excretion is more pronounced than the L-ascorbic acid excretion (Table 11). The data indicate that without D-glucuronolactone feeding little carbohydrate is metabolized via the glucaric acid pathway and that a higher turnover rate of D-glucuronolactone in the ascorbic acid pathway than in the glucaric acid pathway is not the cause of the big difference between D-glucaric acid excretion and L-ascorbic acid excretion in untreated animals.

D-glucuronic acid formed in the liver is the precursor of L-ascorbic acid and D-glucaric acid (Sections 1.1.2 and 1.1.3). For the low excretion of D-glucaric acid as compared with L-ascorbic acid two obvious explanations can be given:
1. The lactonization of endogenous D-glucuronic acid is insufficient, consequently no D-glucuronolactone is available for the terminal step of the glucaric acid pathway. But the condition is that L-ascorbic acid is synthesized via D-glucuronic acid and L-gulonic acid (Pathway B in Fig. 3) and not via D-glucuronolactone (Pathway A in Fig. 3).
2. The lactonization is not rate limiting for D-glucaric acid formation but D-glucuronolactone dehydrogenase, the terminal enzyme of the glucaric acid pathway, is localized different from the glucuronic acid pathway and the ascorbic acid pathway. Of the enzymes catalyzing the conversions from D-glucuronic acid into L-ascorbic acid the terminal enzyme L-gulonolactone oxidase is spe-

cifically involved in the L-ascorbic acid synthesis. It is not found in relation to any other biochemical *in vivo* conversion. However, D-glucuronolactone dehydrogenase seems not to be specific for the glucaric acid pathway. It may be that this enzyme is identical to aldehyde dehydrogenase (EC 1.2.1.3), an hepatic enzyme involved in the conversion of a variety of aldehydes into carboxylic acids (Section 1.1.3). D-glucuronolactone occurs in solution also in the free aldehyde form. The question arises whether the formation of D-glucaric acid has a specific biochemical significance or whether it should be considered as a more or less incidental process which takes place if a sufficient amount of D-glucuronolactone is available in the neighbourhood of the unspecific aldehyde dehydrogenase. In the latter case the glucaric acid pathway is not an intrinsic part of the glucuronic acid system.

It is postulated that the drug-induced stimulation of the glucuronic acid system starts in the glucuronic acid pathway (Section 2.3). The ascorbic acid pathway as well as the xylulose pathway are linked to the glucuronic acid pathway. The increased supply with substrate i.e. D-glucuronic acid results in an enhanced conversion via the pathways just mentioned. This postulate has been extended by Marsh and Reid (1963). They suggest that the increased supply of D-glucuronic acid caused by drug treatment also leads to an enhanced conversion via the glucaric acid pathway. The enhanced D-glucaric acid excretion, however, occurs less generally than the enhanced L-ascorbic acid excretion (see before), indicating that the enhanced D-glucaric acid formation occurs less generally than the enhanced L-ascorbic acid formation. The latter phenomena can be explained as follows. Only if the supply of the substrate D-glucuronic acid surpasses a certain level, it is also converted via the glucaric acid pathway. The higher supply is reached after a single administration of very effective drugs (barbital, chloretone) or after repeated administration of less effective drugs (borneol, phenylbutazone, nikethamide and aminophenazone). The question remains why the very lipid-soluble compounds DDT and thiopental, strong stimulators of the L-ascorbic acid excretion, after repeated administrations do not cause an enhanced D-glucaric acid excretion in the rat. The higher supply of endogenous D-glucuronic acid in order to effect an enhanced D-glucaric acid excretion is needed because of the slight lactonization of the D-glucuronic acid (see discussion under 1; in the xylulose pathway no lactonase is involved) or because of the missing link between the glucuronic acid pathway and the glucaric acid pathway; in that case the glucaric acid pathway is not an intrinsic part of the glucuronic acid system (see discussion under 2).

The fact that a strongly enhanced D-glucuronic acid excretion in urine occurs only during barbital treatment can be explained as follows: D-glucuronic acid has to be considered as an intermediate and not as an endproduct of

the glucuronic acid system. A high supply of D-glucuronic acid is required in order to get a D-glucuronic acid concentration which is sufficient for a considerable diffusion of the acid from the liver cell to the extracellular space. Barbital treatment results in a strong stimulation of the glucuronic acid system, which means a high endogenous D-glucuronic acid supply.

5.4 On the possible relationship between the drug-induced stimulation of the glucuronic acid system and a drug-induced change in glucuronidation

As mentioned earlier (Section 2.3.2) the activity of rat liver UDP glucuronyltransferase is found to be enhanced after treatment of the animal with certain stimulating drugs (aminophenazone, barbital, polycyclic hydrocarbons). The activity was measured *in vitro*, while body-foreign compounds were used as acceptors. It has been put forward that this enhanced activity might result in a stimulation of the glucuronic acid system on the one hand (Touster et al., 1962) and lead to an increased glucuronidation on the other. In this hypothesis it is implied that an UDP glucuronyltransferase plays a role in the glucuronic acid pathway and that this enzyme would be able to transfer the glucuronyl moiety to exogenous acceptors such as drugs and drug metabolites and to a hypothetical intermediary acceptor (Section 1.1.1.1). The correctness of these assumptions, however, has not been proved (Section 1.1.5). If indeed in both types of processes just indicated the same UDP glucuronyltransferase plays a role it would be expected that the enhanced activity of the UDP glucuronyltransferase would occur simultaneously with the enhancement of the *in vivo* activity of the glucuronidating system and that of the glucuronic acid system.

It has been shown recently that stimulation of the glucuronic acid system is evident within 24 hours after administration of the stimulating drugs barbital (Section 5.2.1), phenobarbital and aminophenazone (Hollmann and Neubaur, 1967). This may be concluded from the enhanced excretion of L-ascorbic acid and also of D-glucaric acid in the case of barbital. In the period of time mentioned no increase of UDP glucuronyltransferase can be detected (Zeidenberg et al., 1967; Hollmann and Neubaur, 1967). The increase in the UDP glucuronyltransferase activity can only be detected after treatment with drugs during several (3–4) days. The detection of the UDP glucuronyltransferase was performed with the aid of exogenous acceptors such as drugs and drug metabolites. The data indicate that an enhanced activity in the glucuronic acid system during drug treatment, which is detectable already after 2 or 3 hours, does not directly correlate with the enhanced activity of the UDP glucuronyltransferase involved in the glucuronidation of drugs. The conclusion is that two different transferases are involved or that in the activation of the glucuronic acid system no transferase is involved at all.

UDPGA is a common precursor of the glucuronidation and the various pathways in the glucuronic acid system. As a result of the stimulating action of drugs the supply with UDPGA may be enhanced with as a result an enhanced utilization of this substance in the various pathways including the glucuronidation.

The *in vivo* study of the influence of drugs, known to stimulate the glucuronic acid system, on the glucuronidation has been performed in experimental animals treated with such drugs during several days. The experiments of Remmer (1962) indicate that an enhanced glucuronidation of sulfadimethoxine occurs in rats after treatment with phenobarbital. Under the same circumstances an enhanced glucuronidation of N-acetyl-p-aminophenol has been observed (Büch et al., 1967). The experiments of Yaffe et al. (1966) suggest that the glucuronide forming capacity in hyperbilirubinemic children can be enlarged by treatment with phenobarbital.

Besides an enhanced supply of UDPGA caused by the stimulating drugs, an enhanced activity of UDP glucuronyltransferase or liver growth may also be involved in the enhanced glucuronidation under these circumstances. As discussed before, within the first 24 hours after administration of the stimulating drug (barbital, phenobarbital, aminophenazone) no enhanced UDP glucuronyltransferase activity can be observed and no liver growth occurs (Kunz et al., 1966a), whereas the stimulation of the glucuronic acid system, especially the L-ascorbic acid synthesis, is already evident. In order to get information on a possible connection between the stimulation of the glucuronic acid system and the enhanced glucuronidation on the level of UDPGA formation, the phenomena in question have been studied during the first 24 hours after administration of the stimulating drug. The possible influence of the enhanced UDP glucuronyltransferase activity, as measured *in vitro* with an exogenous compound as acceptor, and of liver growth can be left out of consideration.

Rats were treated with the exogenous glucuronic acid acceptor salicylamide in combination with certain drugs which stimulate the glucuronic acid system. It has been shown by Hänninen (1966) that salicylamide does not cause an enhanced L-ascorbic acid excretion in the 8-hr period after application of the drug. This indicates that stimulation by salicylamide of the glucuronic acid system does not occur in this period yet. For that reason the glucuronidation of salicylamide during 8 hours after the application of this glucuronide-forming compound was studied. Administration of barbital, DDT and nikethamide, which are known to stimulate the glucuronic acid system (Table 10), hardly results in any glucuronide formation. For that reason these compounds were used in these experiments. The rats were given barbital and nikethamide 1 hour before salicylamide administration and DDT 15 hours before salicylamide ad-

TABLE 14 *Effect of some stimulators of the glucuronic acid system on glucuronidation of salicylamide in rats.*

Treatment*	Conjugated D-glucuronic acid μmoles/8-hr urine**			L-ascorbic acid μmoles/8-hr urine**		
	control	treated	P value	control	treated	P value
Sodium barbital, 150 mg/kg	230 ± 40 (6)	158 ± 18 (6)	<0.01	4.9 ± 1.7	14.8 ± 4.8	<0.001
Nikethamide, 125 mg/kg	198 ± 11 (7)	180 ± 46 (7)	>0.25	4.8 ± 1.9	27.1 ± 4.3	<0.001
DDT, 30 mg/kg	271 ± 27 (6)	209 ± 17 (6)	<0.01	8.4 ± 3.5	13.6 ± 8.4	0.05

* Male immature rats, weighing 125–150 g, were given orally sodium barbital, nikethamide or DDT at zero time. Salicylamide was suspended in 0.1% tragacanth solution. Salicylamide was given 600 mg/kg (about 600 μmoles per rat) at 1 hr to the sodium barbital- and nikethamide-treated rats and at 15 hr to the DDT-treated rats. Control animals were given the solvents concerned.

** Collection of urine was started immediately after administration of salicylamide. The figures and P values are expressed as indicated in Table 9.

ministration. No indication was found that an enhanced glucuronidation of salicylamide takes place under the influence of the stimulating drugs (Table 14). Treatment with barbital and DDT even inhibited that glucuronidation. Yet the treatment of the experimental animals with the stimulating compounds in question causes a stimulation of the glucuronic acid system already during the first hours after application. This can be concluded from the increase of the urinary L-ascorbic acid excretion (Table 14) and in the case of barbital from the increase of the urinary D-glucaric acid excretion (Fig. 13). The data indicate that during this period a stimulation of the glucuronic acid system occurs, whereas the glucuronidation is not enhanced. These results in combination with data from the literature show that the stimulation of the glucuronic acid system and the enhanced glucuronidation, both occurring after treatment with drugs, have a different time course and do not appear to be directly related.

It has recently been shown that a lowered UDP glucuronyltransferase activity occurs shortly after the treatment of rats with aminophenazone (Hollmann and Neubaur, 1967) and a lowered UDPG dehydrogenase activity shortly after the administration of barbital to rats (Hollmann and Neubaur, 1967) and guinea pigs (see Table 16). The lowered glucuronidation which could be observed immediately after barbital administration might be easily explained if shortly after barbital treatment also a lowered UDP glucuronyltransferase activity resulting from a lower level or from an inhibition of this enzyme by barbital would occur.

It might be proposed that the lowered glucuronidation is caused by a high utilization of UDPGA through the stimulated glucuronic acid system resulting in a lowered supply of UDPGA for the glucuronidation. In that case one has to postulate that stimulation of the glucuronic acid system takes place in the steps which lead from UDPGA to D-glucuronic acid. If this supposition holds true one might expect that not only after barbital and DDT treatment, but also after nikethamide treatment a lowered glucuronidation occurs.

5.5 Summary

The first subject dealt with in this chapter concerns the length of the latency period between administration of barbiturate and occurrence of an enhanced production and/or excretion of D-glucaric acid and L-ascorbic acid in rats and dogs. It was found that in rats no increase of the hepatic L-ascorbic acid level can be observed 2 hours after i.p. injection of barbital, whereas after a period of 3 hours an increase in this level is evident. It was also observed that in rat the urine obtained during the first 3 hours after i.p. injection of barbital, contains an increased amount of D-glucaric acid and L-ascorbic acid. In view of these data the conclusion seems justified that already within the first 3 hours after i.p. application of barbital the glucuronic acid system is stimulated. It was known already that a time interval of at least 3 hours separates the i.p. injection of phenobarbital from the moment that an increase in drug metabolism on the basis of a *de novo* enzyme synthesis becomes evident (Ernster and Orrenius, 1965). Although both a stimulation of the glucuronic acid system and an increased drug metabolism in question are found after the application of the same kind of compounds, the experiments discussed above indicate that in the liver the first-mentioned stimulation can be detected earlier than the increase in drug metabolism.

In the experiments on dogs a combination of barbital and thiopental was given intravenously; so in these cases the barbiturates served as stimulator and as anaesthetic. The urine was collected directly from the cannulated ureters. The experiments showed that the urinary D-glucaric acid and L-ascorbic acid levels are found to be increased within the first 2 hours after barbiturate application. The stimulation may be caused either by an enhanced enzyme synthesis for which a period of 2 hours (dog) to 3 hours (rat) is sufficient (Section 5.2.1), or by a completely different type of mechanism, not dependent on a *de novo* enzyme synthesis (see in this respect Chapter 7).

So far, the fact that drug treatment causes an enhanced excretion of D-glucaric acid had been described only as far as the rat is concerned (Marsh and Reid, 1963). The fact that the same phenomena can be observed in man,

guinea pig (Chapter 4) and dog (this chapter) shows that the phenomena in question are not confined to a single species.

In rats given cumulative doses of barbital it appears that in order to obtain an enhanced D-glucuronic acid excretion, higher drug levels are needed than those needed to obtain an enhanced excretion of L-ascorbic acid and D-glucaric acid. After stopping drug application much less L-ascorbic acid, D-glucaric acid and D-glucuronic acid is excreted than during the application. After the final dose of barbital, the enhanced D-glucuronic acid excretion is observed for only one day more, whereas the enhanced excretion of the other two acids is observed for more than a week. The pretreatment level of the D-glucuronic acid excretion was very high. It has to be considered that a small D-glucuronic acid response (of the same order as the L-ascorbic acid response) possibly occurring after drug treatment, may be obscured because of the high base level of urinary D-glucuronic acid. The response in D-glucaric acid excretion tends to coincide with that of L-ascorbic acid. The increase in excretion of both substances, although small, is still observed on the 11th day after stopping the barbital application. This prolonged small increase may be related to a prolonged action of the drug; the elimination of barbital from the body is a slow process.

During this study it was found that in individual untreated rats the amount of L-ascorbic acid excreted from day to day in the urine is rather constant. The amounts excreted by different rats of the same strain, however, can vary considerably. It was observed that a positive correlation exists between the barbital-induced increase of L-ascorbic acid excretion and the pretreatment level of excretion.

Not only the effect of barbital, but also the effect of some other drugs on the urinary D-glucaric acid and L-ascorbic acid excretion of the rat was studied. It was found that all compounds tested cause an increase in L-ascorbic acid excretion, whereas an enhanced D-glucaric acid excretion is less common. In the cases that both effects were observed the increase in L-ascorbic acid excretion occurred earlier than the other response and could be brought about by a single dose of the drug in question, whereas an increased excretion of D-glucaric acid became evident only after treatment with the drug during 3 to 4 days. In this respect barbital forms an exception. The same holds for chloretone (Marsh and Reid, 1963).

After treatment of rats during 4 days with some of the compounds studied, the weight of the liver was found to be increased. This increase in weight is supposed to be caused by a hypertrophy of the liver (Section 5.3). The enhanced urinary excretion of L-ascorbic acid and D-glucaric acid, which occurs after some days of drug treatment, might be related to this liver growth. The experi-

ments described in this chapter, however, give an indication that the growth of the liver does not play a definite role in the enhanced excretion of the acids in question.

The enhanced excretion of L-ascorbic acid as found in the rat can be considered as a criterion of the stimulation of the glucuronic acid system. This does not hold for the enhanced excretion of D-glucaric acid, since in some cases an enhanced L-ascorbic acid excretion is found, whereas the D-glucaric acid excretion has not or hardly changed. From the experimental results obtained with rats the conclusion seems justified that the finding of an enhanced D-glucaric acid excretion in this species implies that a strong stimulation has taken place. Possibly, the same holds true in man and guinea pig.

The influence of drugs on the urinary D-glucaric acid excretion in man (Chapter 4) is largely analogous to the effects observed in the animal experiment (this chapter). As for the barbiturates an enhanced excretion of D-glucaric acid is found in man (phenobarbital) as well as in rat, guinea pig and dog (barbital). The influence of aminophenazone and phenylbutazone on the D-glucaric acid excretion in man is also analogous to that found in the rat, whereas salicylic acid (a week stimulating drug) has no effect in this respect in either species. It may be stressed that drugs like aminophenazone and borneol, which are known to cause an enhanced excretion of L-xylulose in pentosuriacs (Enklewitz and Lasker, 1935) also cause an increase of the D-glucaric acid excretion in rats. Furthermore, all the drugs mentioned here cause an enhanced excretion of L-ascorbic acid in rats.

The fact that after the application of a single dose of some drugs a clear response in the L-ascorbic acid excretion is observed, whereas the excretion of D-glucaric acid remains unaltered, probably means that a stimulation of the glucuronic acid pathway has not necessarily the same consequences for all metabolic pathways involved in a further metabolism of D-glucuronic acid. In this context it should be stressed that, according to the experiments of Sadahiro et al. (1966), hepatic D-glucuronolactone dehydrogenase, which catalyzes the terminal step in the D-glucaric acid formation, may be identical with the rather unspecific enzyme aldehyde dehydrogenase (EC 1.2.1.3). This possibility and the fact that under normal conditions carbohydrates are hardly metabolized into and excreted as D-glucaric acid, suggest that, in contrast to the ascorbic acid pathway and the xylulose pathway, the glucaric acid pathway is not an intrinsic part of the glucuronic acid system. On this basis an uncomplicated explanation can be given of the different effects of drugs on the ascorbic acid pathway on the one hand and on the glucaric acid pathway on the other hand. An alternative explanation is that the L-ascorbic acid biosynthesis proceeds via D-glucuronic acid and L-gulonic acid in which case D-glucuronolactone is

not an obligatory intermediate for the L-ascorbic acid synthesis, and that a lactonization of D-glucuronic acid to D-glucuronolactone occurs to a low degree. As discussed in Section 1.1.3 the D-glucaric acid is formed via D-glucuronolactone.

Another pathway, possibly linked to the glucuronic acid system is that leading to the formation of glucuronides. The question arises whether there is a functional link between the enhanced glucuronidation of drugs and drug metabolites, and the stimulation of the glucuronic acid system, as both are observed after administration of compounds of the same nature. A theoretical possibility would be that both phenomena are dependent on an enhanced activity of the same UDP glucuronyltransferase. On the basis of both data found in the literature and the results of our own experiments, this is rather unlikely. An alternative is that both the enhanced glucuronidation and the enhanced formation of the endproducts of the glucuronic acid system are dependent on an enhanced formation of UDPGA in the liver cell. This does not lead to a simple solution either, since it was found that in animals to which a stimulating drug and also a glucuronide acceptor (salicylamide) had been given, no enhanced glucuronidation could be observed during a period of time which was sufficient for the development of a clear stimulation of the glucuronic acid system. If barbital and DDT are used as stimulating drugs, the glucuronidation may even be depressed.

In view of the foregoing it would not seem probable that one and the same mechanism is responsible for the stimulation of the glucuronic acid system and the enhanced formation of glucuronides, which becomes evident only after some days of treatment with the stimulating drugs. The enhanced formation of glucuronides might be explained by an increase in activity of the UDP glucuronyltransferase and also by liver growth. In contrast with the stimulation of the glucuronic acid system, these changes are not found within the first 24 hours after administration of the stimulating drug.

CHAPTER 6

A STUDY ON THE POSSIBLE ROLE OF *DE NOVO* ENZYME SYNTHESIS IN THE DRUG-INDUCED STIMULATION OF THE GLUCURONIC ACID SYSTEM

6.1 Introduction

The stimulating effect of drugs on the biosynthesis of L-ascorbic acid, L-xylulose and D-glucaric acid is probably due to an increased formation of the common intermediate D-glucuronic acid from D-hexose (Sections 2.3 and 4.2.2). As a matter of fact, the suggestion has been made that this increased formation might be the result of a drug-induced elevation of the level of certain liver enzymes involved in the glucuronic acid system.

In studies on the effect of stimulating drugs administered *in vivo* on rat liver enzymes involved in the glucuronic acid system, such as UDPG dehydrogenase and UDP glucuronyltransferase, enhanced activities were found which are possibly due to *de novo* synthesis of these enzymes (Chapter 2). Chloretone (Hollmann and Touster, 1962; Conney et al., 1961) and barbital (Conney et al., 1961) cause an enhanced UDPG dehydrogenase activity. Stimulation of UDPG dehydrogenase activity by 3,4-benzpyrene reported by Arias et al. (1963) was not confirmed by other investigators (Hollmann and Touster, 1962). Carcinogenic hydrocarbons and aminophenazone have no influence on UDPG dehydrogenase activity, but enhance UDP glucuronyltransferase activity. The postulate that an increased level of UDP glucuronyltransferase would imply an enhancement of the metabolism of carbohydrate via the glucuronic acid system is not supported by further observations (Section 5.4). The question whether the enhanced activity of UDPG dehydrogenase could be responsible for the stimulation of the glucuronic acid system by chloretone or barbital has not been definitively answered. Further experiments in this respect are required.

The investigations described in this chapter are aimed at the elucidation of three questions:

1. What is the function of an enhanced UDPG dehydrogenase activity in the stimulation of the glucuronic acid system?
2. Is *de novo* enzyme synthesis, that is synthesis of new enzyme molecules from amino acids, involved in the stimulation of the glucuronic acid system?
3. The results obtained from the experiments described in this chapter raise

the question as to whether the enhanced excretion of D-glucaric acid resulting from the stimulation of the glucuronic acid system can be considered a criterion for the occurrence of drug-stimulated enhanced drug metabolism. This question will be discussed in this chapter too.

6.1.1 Evaluation of the function of the enhanced UDPG dehydrogenase activity of the liver in the stimulation of the glucuronic acid system

As mentioned before (Section 6.1) further experiments have to be performed in order to determine whether an enhancement of the UDPG dehydrogenase activity of the liver, as observed *in vitro*, plays an essential role in the phenomenon indicated as stimulation of the glucuronic acid system (Section 2.3.2). It is known that the enhanced activity of the liver enzyme systems involved in drug metabolism as observed after treatment of experimental animals with certain drugs, is dependent on a *de novo* enzyme synthesis (Ernster and Orrenius, 1965). The notion that a comparison between the influence of certain drugs on the activity of a drug-metabolizing enzyme system on the one hand and on the UDPG dehydrogenase activity on the other hand might be important, is based on the following considerations. First there is the fact that both the stimulation of the glucuronic acid system and of an enhanced drug metabolism can be observed after administration of drugs of the same type. Secondly, the glucuronic acid system may be linked to the glucuronidation, which takes part in drug metabolism. UDPG dehydrogenase catalyzes the formation of UDPGA which is an intermediate in glucuronidation and in the glucuronic acid system (Fig. 1). Thirdly, it is important to know whether an enhancement of the UDPG dehydrogenase activity as a result of drug administration (Section 6.1) is of the same order as the enhancement of the activity of a drug-metabolizing enzyme system in which a *de novo* synthesis is involved. The enzyme system responsible for the demethylation of aminophenazone was chosen as a drug-metabolizing enzyme system to be studied. In the experiments in question it was further determined whether barbital has an influence on the hepatic tyrosine aminotransferase activity. This aspect of the barbital action will be discussed in Section 7.1.1.

Rats and guinea pigs were used as experimental animals. The enzyme activities were measured 24 hours after the administration of the stimulating compound, in this case barbital. After barbital administration the 24-hr urine portions were collected. The levels of D-glucaric acid and L-ascorbic acid (in the rat only) were determined in these urine portions. Since the urinary levels of D-glucaric acid and L-ascorbic acid were found to be enhanced, it was concluded that stimulation of the glucuronic acid system had occurred (Table 15).

TABLE 15 *Effect of barbital treatment** on enzyme activities of guinea pig and rat liver homogenates and on urinary excretion of D-glucaric acid or L-ascorbic acid in rats or guinea pigs.*

Enzyme activity per mg protein	Guinea pigs*			Rats*		
	control	treated	P value	control	treated	P value
UDPG dehydrogenase units	21.3 ± 8.1 (6)	25.7 ± 11.1 (11)	>0.25	16.5 ± 2.4 (6)	18.2 ± 6.1 (6)	0.25
Demethylation 10^3 μmoles/hr	12.1 ± 2.3 (6)	27.3 ± 4.5 (11)	<0.01	10.3 ± 2.1 (6)	18.4 ± 4.6 (6)	<0.01
Tyrosine aminotransferase units	9.2 ± 1.7 (6)	10.4 ± 5.2 (11)	>0.25	7.8 ± 1.5 (6)	6.8 ± 1.1 (6)	>0.10
Urinary excretion*** μmoles/24 hr						
L-ascorbic acid				12.8 ± 8.4 (6)	32.3 ± 18.4 (6)	0.03
D-glucaric acid	0.60 ± 0.32 (6)	5.7 ± 2.2 (11)	<0.01	0.42 ± 0.11 (6)	2.1 ± 0.6 (6)	<0.01

* Female guinea pigs, weighing 300–400 g, and female rats, weighing 180–200 g, were used.

** The animals were given sodium barbital 150 mg/kg i.p. 24 hr before killing; control animals were given saline i.p.

*** Collection of urine was started immediately after injection.

Means with S.D. are shown. Numbers in parenthesis indicate number of animals on which each mean is based. P values were obtained by applying the two-sided Wilcoxon two-sample test.

This stimulation is accompanied with an enhanced demethylation activity of the 9000 *g* supernatants of the liver homogenates obtained by killing the animals. Since enhanced drug metabolism has been shown to be based on *de novo* synthesis of enzymes, this finding implies that an enhanced *de novo* synthesis of the microsomal enzyme systems involved in drug metabolism has taken place. On the other hand no enhanced activity of the UDPG dehydrogenase could be observed. The absence of an increase in the UDPG dehydrogenase activity after treatment with barbital was an unexpected finding.

The half-life time of UDPG dehydrogenase is assumed to be about 4 hours (Hollmann and Neubaur, 1967). As shown by Steiner (1964) the induction of UDPG-glycogen glucosyltransferase — as caused by feeding of starved rats — reaches its maximal value after 3 hours and has disappeared after 20 hours. If a possible induction of the UDPG dehydrogenase activity after barbital treatment would follow a similar pattern, the absence of an increased activity of this enzyme after 24 hours would be understandable. We, however, also studied the UDPG dehydrogenase activity in guinea pigs at various times from 5–72 hours after barbital administration (Table 16). In no case the activity was

TABLE 16 *UDPG dehydrogenase activity of guinea pig liver homogenates at various times after administration of barbital.*

Time after administration*	UDPG dehydrogenase activity per mg protein units		
hr	control	treated	P value
5	28.5 ± 2.9 (6)	24.5 ± 3.8 (6)	0.05
9	15.7 ± 8.0 (6)	23.0 ± 5.3 (6)	0.09
24	21.3 ± 8.1 (6)	25.7 ± 11.1 (11)	>0.25
72	23.5 ± 4.8 (6)	23.8 ± 3.6 (6)	>0.25

* Female guinea pigs, weighing 300–400 g, were given single injections of sodium barbital 150 mg/kg i.p. at zero time. The 72-hr group was given three injections at zero time, at 24 hr and at 48 hr; the control animals were given saline.
The figures and P values are calculated as indicated in Table 15.

found to be enhanced; after a 5-hr period it even appeared to be decreased. Recently such an inhibiting effect of barbital in the rat was reported by Hollmann and Neubaur (1967). These authors ascribe this effect to a primary inhibition of protein synthesis. They too were unable to detect an increase in the hepatic UDPG dehydrogenase activity for various periods up to 48 hours after administration of barbital to rats.

TABLE 17 *Effect of chloretone treatment* on UDPG dehydrogenase activity of rat liver homogenates and on L-ascorbic acid excretion in rat urine.*

Treatment	UDPG dehydrogenase activity per mg protein units			L-ascorbic acid excretion** µmoles/24 hr		
	III control	treated	P value	III control	treated	P value
I 150 mg/kg orally, daily for 6 days	16.1 ± 3.6 (7)	30.8 ± 13.3 (6)	0.03	3.5 ± 2.5 (7)	39.9 ± 21.6 (6)	<0.01
II 150 mg/kg orally, single dose	16.1 ± 3.6 (7)	18.8 ± 6.5 (8)	>0.25	3.5 ± 2.5 (7)	23.3 ± 8.6 (8)	<0.01

* Female rats, weighing 180–220 g, were used. Groups I and II were killed 24 hr after the last dose and 20 hr after the single dose respectively. The animals in groups I and II were compared with control animals (group III), which were killed at the same time.
** Collection of urine was started immediately after the last dose.
Figures and P values are calculated as indicated in Table 15.

According to Conney et al. (1961) and Hollmann and Touster (1962) the UDPG dehydrogenase activity is enhanced after pretreatment with chloretone. These investigators administered the drug daily to rats during 7 days. It was thought important to investigate whether also after treatment with a single dose of chloretone an enhanced UDPG dehydrogenase activity would occur. The following experiment was performed (Table 17). From three groups of rats group I was treated daily for 6 days with chloretone, group II received a single dose of this drug, whereas group III served as a control. In group II the enzyme activity was determined 20 hours after the dose of chloretone, in group I 24 hours after the last dose. Moreover, the amount of urinary L-ascorbic acid, excreted during 24 hours and 20 hours in the groups I and II respectively, was determined. The measured enzyme activities and urinary excretions were compared with those of the control group (III). As may be concluded from the increased L-ascorbic acid excretion, a stimulation of the glucuronic acid system occurs after repeated administration of chloretone as well as after a single dose of this drug. An enhanced activity of the UDPG dehydrogenase, however, was only observed in the chronically treated animals (group I). From these data it may be concluded that the stimulation in the L-ascorbic acid production observed within the first 24 hours after administration of the drug is not dependent on an enhanced UDPG dehydrogenase activity. The D-glucaric acid excretion is also considerably enhanced within 24 hours after administration of chloretone to rats (Marsh and Reid, 1963). Since the early responses of L-ascorbic acid and D-glucaric acid as compared with the responses obtained after continuous treatment are quite considerable already, it is unlikely that an increase in the UDPG dehydrogenase activity plays an important role in the overall phenomenon of the drug-induced stimulation of the glucuronic acid system.

6.1.2 Is a de novo *protein synthesis involved in the drug-induced stimulation of the glucuronic acid system?*

From experiments described in Section 5.2.1 it appears that in rats stimulation of the glucuronic acid system occurs within 3 hours after barbital administration. From experiments with dogs (Section 5.2.1) it also appears that stimulation occurs within 2 hours after administration of barbiturates. It has been shown in the Sections 5.4 and 6.1.1 that *de novo* synthesis of UDP glucuronyltransferase and UDPG dehydrogenase respectively, is not involved in the stimulation occurring within a few hours after drug administration. The possibility has to be considered that although no indication has been found in this direction (Section 2.3.2) other enzymes are involved in the stimulation and that *de novo* synthesis of these enzymes occurs in the first few hours after administration of a barbiturate.

In order to evaluate the role of such a *de novo* synthesis the effects of inhibitors of protein synthesis were studied. The inhibitors used were puromycin and actinomycin D. Puromycin inhibits the transfer of the amino acids from soluble ribonucleinic acid (RNA) to protein (Yarmolinsky and de la Haba, 1959). It has been shown that the inhibition of protein synthesis by puromycin occurs practically immediately (15 min after i.p. injection) (Guidice et al., 1964). Actinomycin D is supposed to inhibit deoxyribonucleinic acid (DNA)-directed RNA synthesis (Traketellis et al., 1964). For actinomycin D it has been shown that 30 min after i.p. injection protein synthesis is inhibited (Nakazato et al., 1965).

In our experiments the question was studied whether puromycin and actinomycin D influence the stimulation of the glucuronic acid system, as caused by barbital.

Rats were injected with puromycin or actinomycin D in quantities high enough to prevent protein synthesis within the first 6 hours after barbital administration. Enhanced urinary excretion of L-ascorbic acid and D-glucaric acid was used as an indication for the occurrence of stimulation (Table 18). The data show that puromycin and actinomycin D do not prevent the effect of barbital. It has been shown by de Matteis (1964), that in actinomycin D-treated rats the stimulating effect of 2-allyl-2-isopropylacetamide on the L-ascorbic acid excretion is still very pronounced, while the porphyria-inducing effect of the same drug — an effect which is dependent on *de novo* enzyme synthesis — is abolished. The results obtained indicate that *de novo* synthesis is not involved in the stimulation of the glucuronic acid system, as caused by drugs such as barbital and 2-allyl-2-isopropylacetamide. Assuming that the stimulating drugs, although they have different chemical structures and pharmacological properties, act by the same mechanism, the same holds for stimulation caused by other drugs.

6.1.3 The enhanced excretion of D-glucaric acid in animals during treatment with drugs as an indication of an enhanced drug-metabolizing capacity of the liver

According to Burns et al. (1963) an enhanced excretion of L-ascorbic acid in rats during treatment with certain drugs may be considered as an indication of enhanced drug metabolism in the liver. This enhanced excretion of L-ascorbic acid is caused by an accelerated metabolism of carbohydrates via D-glucuronic acid (Chapter 2). Since man does not synthesize L-ascorbic acid, it is a relevant question whether another product formed via D-glucuronic acid and excreted in the urine might serve as an indication of the stimulation of the glucuronic acid system and/or induction of enhanced drug metabolism in patients. Theoretically D-glucaric acid might serve as such an indicator (Section 4.1). The

TABLE 18 *Barbital-stimulated urinary excretion of L-ascorbic acid and D-glucaric acid in rats treated with puromycin and actinomycin D.*

Treatment*		3 hr Before experiment	First 3 hr during experiment		Second 3 hr during experiment	
		μmoles/3 hr	μmoles/3 hr	P value	μmoles/3 hr	P value
I Barbital with puromycin	(6)	L-ascorbic acid 0.68 ± 0.38	1.67 ± 0.53	0.03	3.66 ± 0.95	0.03
		D-glucaric acid 0.044 ± 0.009	0.143 ± 0.036	0.03	0.174 ± 0.022	0.03
II Barbital with actinomycin D**	(6)	L-ascorbic acid 0.60 ± 0.41	1.02 ± 0.31	0.06	5.23 ± 2.64	0.03
		D-glucaric acid 0.029 ± 0.006	0.049 ± 0.017	>0.10	0.165 ± 0.104	0.03
III Barbital	(8)	L-ascorbic acid 0.80 ± 0.43	2.11 ± 1.43	0.03	3.75 ± 1.43	0.03
		D-glucaric acid 0.035 ± 0.008	0.096 ± 0.045	0.03	0.222 ± 0.066	0.03

* Male weanling rats, weighing 45–60 g, were divided into three groups. After collecting urine over a 3-hr period the rats were treated as follows:

Group I was injected with sodium barbital 140 mg/kg i.p. at zero time, together with puromycin 30 mg/kg. The puromycin injections were repeated every hour.

Group II was injected twice with actinomycin D 800 μg/kg i.p. at zero time and at 3 hr. The group was injected with sodium barbital 140 mg/kg i.p. at 30 min.

Group III was treated as group I except that puromycin was omitted. Urine was collected in two consecutive 3-hr periods.

** Treatment of a group of six rats with only actinomycin D did not influence significantly urinary excretion of the acids during the first 6 hr after injection.

Numbers in parenthesis indicate number of animals in each group. Means with S.D. are shown. P values were obtained by applying the two-sided Wilcoxon signed-rank test. The two 3-hr intervals after treatment were compared with the 3-hr interval before treatment. Comparison of the excretion of the acids of groups I and II with group III did not show significant differences (P value < 0.05) in the second 3-hr interval and in the control 3-hr interval (two-sided Wilcoxon two-sample test).

basic assumption is, that the enhanced drug metabolism and the enhanced metabolism of carbohydrates via the glucuronic acid system are functionally tied up or at least correlated in time. Further analysis, however, leads to the following results.

1. In animal experiments an enhanced carbohydrate metabolism via D-glucuronic acid manifests itself much more clearly in an enhanced excretion of L-ascorbic acid than in a change in the excretion of D-glucaric acid. Some drugs which stimulate drug metabolism, such as barbital (Table 10) and chloretone (Marsh and Reid, 1963), cause already after a single dose an enhanced excretion of D-glucaric acid in the rat, whereas other stimulating drugs such as aminophenazone, borneol, nikethamide and phenylbutazone have such an effect only after a repeated administration during some days. A third group of stimulating drugs such as DDT (Table 13) and 3-methylcholanthrene (Marsh and Reid, 1963) does not cause a clear change in D-glucaric acid excretion, even after repeated dosing during several days. An important enhancement of the urinary L-ascorbic acid excretion in the rat follows a single dose of any of these drugs.

2. Contrary to the enhanced drug metabolism, the stimulation of carbohydrate metabolism via D-glucuronic acid appears not to be caused by a *de novo* enzyme synthesis (Section 6.1.2), which indicates that different basic processes are involved.

Recapitulating, an enhanced D-glucaric acid excretion does not necessarily follow the administration of stimulators of drug metabolism, whereas the biochemical mechanisms involved in an enhanced drug metabolism and in an enhanced D-glucaric acid excretion have little in common. It is even questionable whether the synthesis of D-glucaric acid and that of L-ascorbic acid are related in the same way with the glucuronic acid pathway (Section 5.3.2). Therefore, the supposition that the enhanced excretion of D-glucaric acid might be considered an indication of an increased drug-metabolizing capacity in the liver does not seem to be justified. This holds for animal experiments and there is no reason to suppose that the same would not hold too for the clinical use of drugs.

6.2 Summary

Several investigators put forward the possibility that an enhanced *de novo* synthesis of liver UDPG dehydrogenase caused by drugs might be responsible for the enhanced biosynthesis and excretion of products of the glucuronic acid system. In the present study this possibility was further tested. First it was determined whether the activity of liver UDPG dehydrogenase is enhanced after treatment with barbital, a drug which strongly stimulates the glucuronic

acid system, and whether this enhancement is in the same order of magnitude as the increase in activity of an inducible drug-metabolizing enzyme system. It has also been investigated whether the activity of D-glucuronolactone dehydrogenase, the enzyme concerned in the terminal step of the glucaric acid pathway, is enhanced. The oxidative demethylation of aminophenazone was measured since the responsible enzyme is a good example of an inducible drug-metabolizing enzyme system, in which enhanced *de novo* synthesis is involved.

After barbital treatment of rats and guinea pigs no enhanced UDPG dehydrogenase activity was observed, whereas the demethylation activity was enhanced and stimulation of the glucuronic acid system was evident. From the foregoing it follows that an increase of the UDPG dehydrogenase activity is not the primary cause of the stimulation of the glucuronic acid system. On the other hand an enhanced UDPG dehydrogenase activity, which occurs after drug treatment during several days, may be involved in causing an enhanced excretion of products of the glucuronic acid system. A single dose of chloretone as well as the administration of this drug during several days causes an enhanced excretion of L-ascorbic acid, indicating that the glucuronic acid system is stimulated; the activity of UDPG dehydrogenase, however, is enhanced only after treatment with this drug during several days.

As discussed earlier (Section 5.4) an enhanced UDP glucuronyltransferase activity, which is measured with the aid of exogenous substances as acceptors, appears not to be primarily involved in the stimulation of the glucuronic acid system.

The stimulation caused by barbital is not prevented by treatment with actinomycin D or puromycin. A similar observation was made by de Matteis (1964), who observed that actinomycin D does not prevent the stimulating effect of 2-allyl-2-isopropylacetamide on the formation of L-ascorbic acid. If, as is probable, most stimulating drugs, although they have different chemical structures and pharmacological properties, act by the same mechanism as far as enhancement of drug metabolism is concerned, the same may hold true for stimulation of the glucuronic acid system. The results obtained with drugs as actinomycin D and puromycin, which inhibit the protein synthesis, indicate that, contrary to the stimulation of drug metabolism, the stimulation of the glucuronic acid system is not dependent on a *de novo* enzyme synthesis.

The question was asked whether the enhanced urinary D-glucaric acid excretion occurring in animals during drug treatment can be used as an indication of an existing enhanced drug-metabolizing capacity in the liver of man or animal involved. In view of the results of the experiments discussed in this chapter and of the experiments described in Chapter 5, the answer in this question can only be negative.

CHAPTER 7

THE BASIC CHEMICAL PROCESSES POSSIBLY INVOLVED IN THE STIMULATION OF THE GLUCURONIC ACID SYSTEM AS CAUSED BY DRUGS

7.1 Introduction

In this chapter the emphasis will be put on the possible, primary biochemical processes involved in the stimulation of the glucuronic acid system as caused by drugs. The following aspects will be studied:

1. Are hormonal processes involved in the stimulating effect of drugs on the glucuronic acid system?

2. It is known that a variety of drugs cause stimulation of the pentose phosphate pathway. The stimulation of this pathway is accompanied by liver growth and follows a change in the NADP redox state in the cytoplasm of the liver cells. A comparison of the stimulation of the pentose phosphate pathway with that of the glucuronic acid system is made. This comparison may be useful for further analysis of the stimulation of the glucuronic acid system by drugs.

7.1.1 The involvement of hormones in the stimulation of the glucuronic acid system

With respect to a hormonal action several possibilities can be considered: The hormones are administered to the animals acting directly on the glucuronic acid system or the hormones are of endogenous origin and released under the influence of the drug. In the latter case the drugs stimulate the glucuronic acid system in an indirect way. Another possibility is that hormones have an influence on the magnitude of the response as caused by stimulating drugs. As far as the application of hormones, especially steroid hormones, is concerned, there is evidence that after dosage of such compounds a stimulation of the glucuronic acid system takes place, manifesting as increased excretion of L-ascorbic acid (Section 2.2). These observations were based on the application of doses of hormones which must be considered as very high, as compared to the quantity of hormones normally involved in the biological regulation processes. Moreover, the responses of the glucuronic acid system upon hormone treatment were weak.

A direct approach to the involvement of endogenously released hormones in

the stimulation phenomenon is the study of the influence of drugs on the glucuronic acid system in animals deprived of certain endocrine glands. Application of chloretone and barbital to adrenalectomized animals still results in a clear increase in the L-ascorbic acid excretion (Burns et al., 1957). On the other hand it is possible to stimulate the excretion of corticoid hormones in animals by placing them in certain stress situations without the involvement of drugs. It has been shown that in such a stress situation the activity of certain enzymes e.g. tyrosine aminotransferase in the liver tissue is enhanced (Kenney and Flora, 1961). It is known that after application of hydrocortisone the activity of this enzyme is also enhanced. The question arises whether in a stress situation in which the adrenals are involved an enhanced excretion of L-ascorbic acid takes place and whether the activity of hepatic enzymes involved in glucuronic acid metabolism e.g. UDPG dehydrogenase and D-glucuronolactone dehydrogenase is enhanced. It is known that under the influence of certain steroid hormones and of certain stress situations to which animals are subjected the activity of the drug-metabolizing enzyme systems can be enhanced (Schenkman et al., 1967; Furner and Stitzel, 1968). The question arises whether under stress condition to which the animals in our experiment were subjected an increase in the drug-metabolizing capacity takes place too.

Experiments were performed with rats. The animals were treated with the stress-inducing agent celite (a soluble diatomaceous earth, which cannot be considered as a drug in the usual sense). Studied were the L-ascorbic acid and D-glucaric acid excretion, the activity of the liver enzymes UDPG dehydrogenase and D-glucuronolactone dehydrogenase and the drug-demethylation activity of liver tissue. In order to check whether a stress situation has been reached in the same experiment the tyrosine aminotransferase activity was measured. As said before an enhanced tyrosine aminotransferase activity follows the stress situation under study. The results are summarized in Table 19. It is observed that during a celite-induced stress no enhanced excretion of L-ascorbic acid or D-glucaric acid takes place in the rats. The activity of hepatic UDPG dehydrogenase and D-glucuronolactone dehydrogenase is not enhanced, nor is the drug-demethylation activity of the liver microsomes. The fact that the activity of the tyrosine aminotransferase is enhanced indicates that a stress situation was really reached. The experimental results (Table 19) and the results obtained by Burns et al. (1957) on adrenalectomized rats allow the conclusion that stimulation of the adrenals which cause the production and release of corticoid hormones does not result in an enhanced activity in the glucuronic acid system, nor in an enhanced activity of the drug-demethylation system of the liver. Through experiments, described in Table 15, it was shown that barbital does not influence the activity of hepatic tyrosine amino-

TABLE 19 *Effect of celite treatment* on enzyme activities of rat liver homogenates and on urinary excretion of L-ascorbic acid and D-glucaric acid in rats.*

Enzyme activity per mg protein	control	treated	P value
UDPG dehydrogenase units	16.5 ± 2.4 (6)	18.0 ± 4.3 (6)	>0.10
D-glucuronolactone dehydrogenase 10^2 µmoles/hr	2.3 ± 0.3 (6)	2.6 ± 0.5 (6)	>0.25
Demethylation 10^3 µmoles/hr	10.3 ± 2.1 (6)	9.8 ± 2.0 (6)	>0.25
Tyrosine aminotransferase units	7.8 ± 1.5 (6)	31.5 ± 9.7 (6)	<0.01
Urinary excretion** µmoles/24 hr			
L-ascorbic acid	12.8 ± 8.4 (6)	10.7 ± 4.2 (6)	>0.25
D-glucaric acid	0.42 ± 0.11 (6)	0.51 ± 0.14 (6)	>0.25

* Female rats, weighing 180–200 g, were given celite 60 mg/kg i.p. 24 hr before killing; control animals were given saline i.p.

** Collection of urine was started immediately after injection.

Means with S.D. are shown. Numbers in parenthesis indicate number on which each mean is based. P values were obtained by applying the two-sided Wilcoxon two-sample test.

transferase. It may be concluded that the enhanced activity of the glucuronic acid system and that of the drug-demethylation system as observed after treatment of animals with barbital, cannot be ascribed to a stress situation induced in these animals by barbital. Probably the same holds true for other drugs.

The possible significance of the hypophysis for the stimulation of the glucuronic acid system by drug treatment has also been analyzed. Burns et al. (1957) report that in hypophysectomized rats treated with chloretone or barbital no enhanced excretion of L-ascorbic acid in the urine takes place. Hollmann and Touster (1962) observed a slightly increased excretion of L-ascorbic acid in hypophysectomized rats treated with chloretone, barbital and 3-methylcholanthrene. Klinger et al. (1965) found a clear, though subnormal enhancement in the excretion of L-ascorbic acid in the urine in hypophysectomized rats after treatment with barbital. In our study the L-ascorbic acid excretion as well as the D-glucaric acid excretion in hypophysectomized rats under the influence of barbital was studied. The results, summarized in Table 20, show that there is an enhanced excretion of both products after drug treatment, which means that the hypophysis is not essentially involved in the stimulation of the glucuronic acid system by drugs.

TABLE 20 *Effect of barbital treatment* on L-ascorbic acid and D-glucaric acid excretion in alloxan-diabetic, hypophysectomized and parathyroidectomized rats.*

Pretreatment**	Urinary excretion*** µmoles/24 hr					
	L-ascorbic acid			D-glucaric acid		
	control	barbital	P value	control	barbital	P value
Alloxan diabetic	7.2 ± 4.2 (7)	24.6 ± 9.5 (5)	<0.01	0.27 ± 0.16 (7)	1.15 ± 0.54 (5)	<0.01
Hypophysectomized	1.0 ± 0.6 (7)	4.8 ± 2.2 (7)	<0.01	0.11 ± 0.05 (6)	1.63 ± 0.52 (6)	<0.01
Parathyroidectomized	0.9 ± 0.6 (6)	3.4 ± 1.9 (6)	<0.01	0.23 ± 0.12 (6)	1.02 ± 0.40 (6)	<0.01

* Male rats, weighing 180–200 g, were used. Two groups of rats deprived of the same hormonal centre were given injections of sodium barbital 150 mg/kg i.p. and saline i.p. respectively.

** Alloxan (40 mg/kg) was given i.v. After 48 hr it was checked that glucosuria occurred and if so, the rats were given barbital or saline.

Hypophysectomy and parathyroidectomy were performed under light ether anaesthesia. About 3 hr after anaesthesia the rats were given barbital or saline. Moreover, the parathyroidectomized rats were given 3 ml 3% calcium lactate about 10 hr after anaesthesia.

*** Collection of urine was started immediately after injection of sodium barbital.

The figures and P values are calculated as indicated in Table 19.

TABLE 21 *Sex difference in L-ascorbic acid response of rats to drug treatment.**

Treatment	Urinary excretion of L-ascorbic acid** µmoles/24 hr/100 g rat					
	before treatment			after treatment		
	male	female	P value	male	female	P value
Niketamide, 150 mg/kg orally	1.9 ± 0.8 (6)	1.3 ± 0.6 (8)	>0.10	13.9 ± 5.9	8.1 ± 2.8	0.03
DDT, 30 mg/kg orally	1.4 ± 0.8 (7)	1.4 ± 0.6 (8)	>0.25	4.9 ± 2.1	2.2 ± 0.6	0.03
Chloretone, 150 mg/kg orally	2.3 ± 0.7 (5)	1.4 ± 0.9 (7)	0.04	33.7 ± 5.9	20.4 ± 3.6	<0.01
				Increase***:		
				31.4 ± 5.8	19.1 ± 3.6	<0.01

* Young adult male and female rats of the same age, weighing 280–320 g and 190–210 g respectively, were used.
Niketamide was given in two doses: at zero time and at 8 hr. DDT was given in a single dose. Chloretone was given in two doses: at zero time and at 24 hr.
** Collection of urine was started immediately after first dose; only after chloretone treatment urine was collected after last dose.
*** Since in this case the untreated male and female rats show a significant difference, the increase in L-ascorbic acid excretion (value after treatment minus value before treatment) was tested.
Means with S.D. are shown; numbers in parenthesis indicate number of animals on which each mean is based. P values were obtained by applying the two-sided Wilcoxon two-sample test.

The significance of the gonades was also studied. Klinger et al. (1965) observed that in male rats which were adrenalectomized and castrated an enhanced excretion of L-ascorbic acid takes place after barbital treatment. In our study the response of the L-ascorbic acid excretion in male rats to nikethamide, DDT, chloretone and barbital was compared with that in female rats (Table 21; Fig. 19; see also Aarts, 1968). The same was done for the D-glucaric acid excretion during barbital treatment (Fig. 19). The response to drug treatment was stronger in male rats. Pretreatment of castrated males with estradiol and of castrated females with testosterone propionate followed by treatment with barbital gave the following results: the response of the L-ascorbic acid and

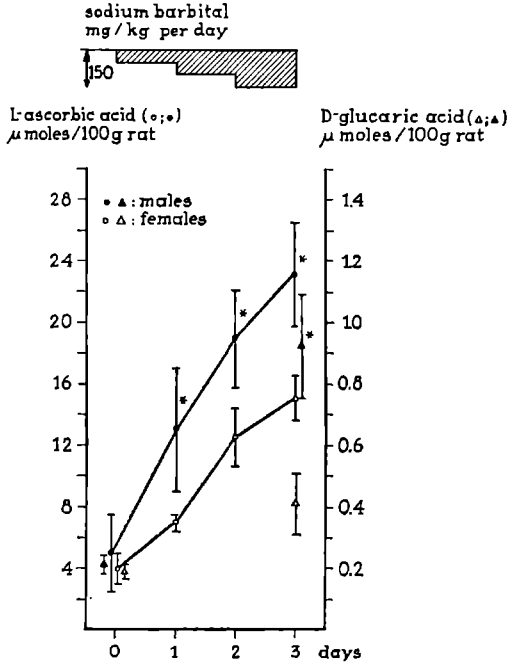

FIG. 19 *Effect of barbital on the urinary excretion of L-ascorbic acid and D-glucaric acid in male and female rats.*

Rats were treated orally with sodium barbital as shown in figure. Each symbol corresponds with 24-hr urine collected after drug administration. Each value represents mean ± S.D. for 8 rats. Asterisk denotes a significant difference between males and females (P value < 0.05; two-sided Wilcoxon two-sample test).

Note Both the increase in L-ascorbic acid excretion and the increase in D-glucaric acid excretion after treatment with sodium barbital are significantly greater in males than in females.

D-glucaric acid excretion is stronger in castrated testosterone-treated females than in castrated estradiol-treated males (Fig. 20). The conclusion may be that, although sex and sex hormones have an influence on the degree of stimulation

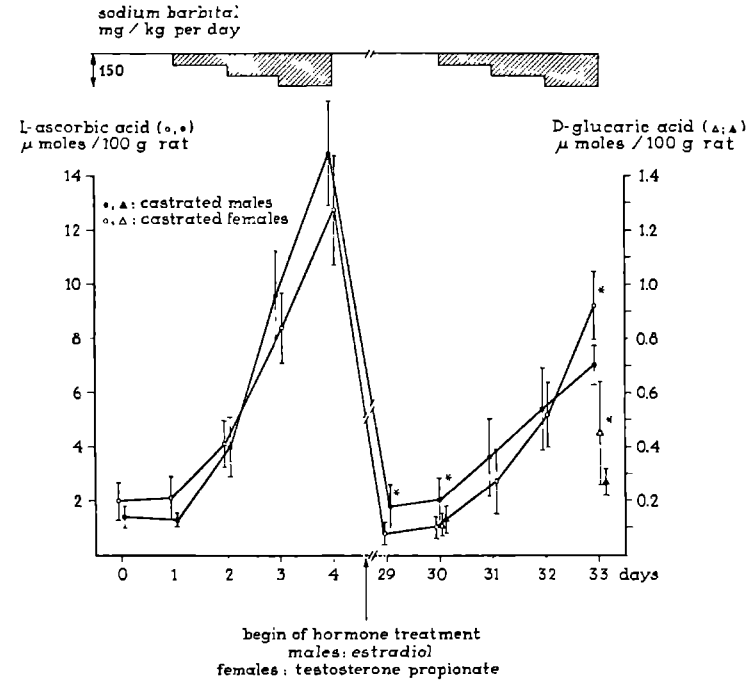

FIG. 20 *Effect of barbital on the urinary excretion of L-ascorbic acid and D-glucaric acid in castrated male and female rats before and after treatment with sex hormones.*

Hormone treatment was started as indicated by arrow and continued during the whole experiment. To the castrated males estradiol and to the castrated females testosterone propionate was given intramuscularly every other day in doses of 2 μg in 0.1 ml sesame oil.
Further conditions are similar to those described in Fig. 19. Each symbol corresponds with 24-hr urine collected after drug administration. Each value represents mean ± S.D. for 8 rats. Asterisk denotes a significant difference.

Note After castration no significant sex difference in the barbital-caused increase in L-ascorbic acid excretion is observed (see first section of the curves and compare with Fig. 19). In the hormone-treated animals the values after administration of 150 mg/kg sodium barbital again differ significantly, but now the testosterone-treated females show the higher L-ascorbic acid and the higher D-glucaric acid excretion (see second section of the curves and compare with Fig. 19).

of the glucuronic acid system as caused by drug treatment, this stimulation is not strictly dependent on the hormones. This holds true especially since in male rats which were castrated and adrenalectomized a response was still to be observed after treatment with barbital (Klinger et al., 1965).

The significance of the thyroid and parathyroid glands and the pancreas for the enhanced L-ascorbic acid and D-glucaric acid excretion after barbital in rats was studied too. In Table 19 it is shown that after thyroid- and parathyroidectomy there is still a clear response to be observed. Klinger et al. (1966) also report an enhanced excretion of L-ascorbic acid in thyroidectomized rats treated with barbital. In rats treated with alloxan, in order to eliminate the β-cells in the pancreas, the stimulation of the glucuronic acid system after drug treatment takes place (Table 20).

The final conclusion may be that the stimulation of the glucuronic acid system in the liver brought about by drugs is not mediated by the products of the various endocrine glands studied.

7.1.2 Comparison of the drug-induced stimulation of the glucuronic acid system with the drug-induced stimulation of the pentose phosphate pathway

Kunz et al. (1966a, b) studied the influence of drugs on the liver growth in mice and the biochemical effects which accompany this growth. One of the studied effects was the stimulation of the pentose phosphate pathway. Since certain aspects of this stimulation may be analogous to the stimulation of the glucuronic acid system, this study may be discussed here in some detail.

In the study in question, barbiturates, chlorcyclizine, nikethamide, DDT, halothane and carcinogenic hydrocarbons were used. It is known that in the rat these substances stimulate drug metabolism as well as the biosynthesis of L-ascorbic acid (Table 1; Section 5.3.1). During treatment of mice with the drugs mentioned the metabolism of D-glucose via the pentose phosphate pathway increases. The redox state of the cytoplasmic NADP system in the liver cell is found to be increased. During treatment not only the liver weight but also the phospholipid, the protein, the RNA and the DNA amount per gram wet liver increases.

The carcinogenic hydrocarbons are exceptional in the sense that these compounds only cause an increase in the amount of proteins and RNA, whereas liver growth is absent and no change of the redox state of the NADP system, no stimulation of the pentose phosphate pathway and no change in the phospholipid and DNA amount occur (Kunz et al., 1966b). It is known that the carcinogenic hydrocarbons stimulate the glucuronic acid system, as follows from the increased L-ascorbic acid biosynthesis occurring after treatment of

rats with these compounds (Table 1). The last mentioned data make it questionable whether stimulation of the pentose phosphate pathway and stimulation of the glucuronic acid system are directly interconnected.

In mice treated with phenobarbital the sequence of the biochemical changes mentioned above was studied (Kunz et al., 1966b). It was found that the changes in phospholipid, protein and RNA content only occur after a latency time of about 48 hours; the changes in incorporation of labeled precursors into free nucleotides, RNA, DNA, proteins and lipids occur after a latency time of about 24 hours. Stimulation of the pentose phosphate pathway is evident already after a latency time of about 12 hours. It is interesting that within a few hours after administration of the drug the level of $NADPH_2$ of the liver cell falls, while the NADP level remains either unchanged or slightly rises. Consequently, the redox state $NADP/NADPH_2$ increased markedly. Metabolism of carbohydrates via the pentose phosphate pathway is linked to the formation of $NADPH_2$. A hypothesis for the drug-induced stimulation of the pentose phosphate pathway may be formulated as follows. The drug causes a shortage of $NADPH_2$. The cell may compensate for this shortage by an enhanced activity of the pentose phosphate pathway. Kunz et al. (1966b) treated mice with phenobarbital, a stimulator of the glucuronic acid system as well as of the pentose phosphate pathway. They conclude that in the liver not only an increase in the redox state of the cytoplasmic NADP system but also in that of the NAD system occurs, since during phenobarbital treatment the concentration ratio of α-glycerol phosphate/dihydroxyacetone phosphate, of lactate/pyruvate and of malate/oxalacetate is decreased. The enzymes involved, i.e. glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase, catalyze the conversion of the substrate and NAD to oxidized substrate and $NADH_2$ as well as the reverse of it.

The glucuronic acid system is linked to $NADH_2$ formation. During the formation of UDPGA hydrogen is transferred to NAD. $NADH_2$ is also formed during the biosynthesis of D-glucaric acid and of D-xylulose via D-glucuronic acid. Analogous to the hypothesis concerning a possible relationship between the shortage of $NADPH_2$ caused by drugs and the attendant stimulation of the pentose phosphate pathway, the following may be proposed as regards the stimulation of the glucuronic acid system by drugs. The drugs primarily induce an increase of the redox state of the cytoplasmic NAD system, viz. a shortage of $NADH_2$. As a reaction upon this shortage an enhanced metabolism of D-hexose via the glucuronic acid system occurs.

In connection with this hypothesis it would be interesting to study the following points:

1. To which degree is the UDPGA formation influenced by the redox state of the NAD system?

2. Is a change in the redox state of the cytoplasmic NAD system of the liver cells during treatment of animals with drugs stimulating the glucuronic acid system a rule and does this change occur as early after drug administration as the stimulation of the glucuronic acid system?

7.2 *Summary*

The mechanism by which drugs stimulate the glucuronic acid system is not known. The phenomenon is not effected via hormonal centres as the adrenals, hypophysis, pancreas, gonades, glandulae thyreoideae or parathyreoideae. Nevertheless some hormones may influence the degree of the stimulation (Section 7.1.1). The kidneys do not play an essential role in the mechanism (Section 2.3). Many data point to the glucuronic acid system in the liver as the main object for the stimulation by drugs (Sections 2.3.1, 4.1 and 4.2.2). The phenomenon appears not to be directly connected with the enhanced capacity for glucuronidation which may follow drug treatment (Section 5.4). Many drugs stimulate both the glucuronic acid system and the pentose phosphate pathway. There are no indications, however, that these phenomena are directly interrelated (Section 7.1.2). In contrast to drug-induced enhanced drug metabolism the drug-induced stimulation of the glucuronic acid system appears not to be dependent on *de novo* protein synthesis (Section 6.1.2). An activation *in vitro* of enzymes of the glucuronic acid system by drug treatment has not been observed. Nor were signs of such an enzyme activation found in liver preparations obtained from animals a few hours after drug treatment (Sections 2.3.2, 5.4 and 6.1.1). It remains possible that *in vivo* the stimulating drugs act by enzyme activation or by a facilitation of either the substrate transport or the interaction between enzyme and substrate, but that as a result of the destruction of the cell these effects cannot be observed *in vitro*.

In future studies on the mechanism of the stimulation phenomenon the following aspects will deserve special attention:

1. The experimental data suggest that the stimulating drugs exert their influence on one of the enzymatic steps, probably the rate-limiting step, involved in the conversion of UDPG to D-glucuronic acid (Section 2.3.1). It should be studied whether the rate-limiting step in the sequence of enzymes leading from UDPG to D-glucuronic acid is located before or after the UDPGA formation.
2. Taking into account the possibility that the stimulation may be effectuated by a facilitation of the UDPGA formation, the following should be considered. During treatment with barbital the redox state of the NAD system in the cytoplasm is shifted towards the oxidized site (Kunz et al., 1966b; Section 7.1.2). It should be investigated whether this phenomenon can be generally observed

after treatment with drugs stimulating the glucuronic acid system. The formation of UDPGA along various pathways of the glucuronic acid system are associated with a production of $NADH_2$. The enhanced metabolism of D-hexose phosphates via the glucuronic acid system might be tied up to the drug-induced change in the NAD redox state of the cytoplasm of the liver cells.

3. Taking into account the possibility that the stimulation of the glucuronic acid system is effected by a facilitation of the formation of D-glucuronic acid from UDPGA (Section 5.4) the following can be said. The biochemical data on the formation of D-glucuronic acid from UDPGA *in vivo* are far from complete. Conclusive experimental data should be obtained in order to answer the questions whether D-glucuronic acid is formed from UDPGA via UDP glucuronyltransferase and β-glucuronidase or via pyrophosphatase-phosphatase (Section 1.1.1). Subsequently, direction is given to the study on the influence of drugs on the enzyme systems involved in the formation of D-glucuronic acid from UDPGA.

SUMMARY

A review is given of the literature on the metabolism of D-glucuronic acid *(Chapter 1)* and on the influence of drugs on that metabolism *(Chapter 2)*. It is followed by a report of the materials and methods used *(Chapter 3)* in the investigations presented in this thesis. The results of these investigations can be summarized as follows:

Chapter 4

1. During barbital treatment an enhanced D-glucaric acid excretion as well as an enhanced drug metabolism (demethylation of aminophenazone) takes place in guinea pigs. Within 24 hours after a single application of the drug the D-glucaric acid excretion is enhanced and levels off to the original value in the 5 days following drug administration.

2. The body pool of D-glucaric acid in guinea pigs is of the same order as the amount of D-glucaric acid excreted in the 24-hr urine. Treatment with barbital leads to a 24-hr excretion of D-glucaric acid which largely exceeds the body pool, indicating that the enhanced D-glucaric acid excretion is the result of an enhanced production.

3. During the treatment of rats with barbital the levels of D-glucaric acid in liver and urine are enhanced. It has been made acceptable that these enhanced levels are the result of an effect taking place in the liver.

4. Treatment of guinea pigs with barbital does not result in an enhanced activity of hepatic D-glucuronolactone dehydrogenase, the enzyme involved in the final step of the D-glucaric acid formation. Barbital treatment has neither an influence on the D-glucaric acid excretion in guinea pigs loaded with D-glucuronolactone nor on the D-mannaric acid excretion in guinea pigs loaded with D-mannuronolactone. The results are in agreement with the hypothesis that the enhanced D-glucaric acid excretion is the result of a drug-induced stimulation of the glucuronic acid pathway.

5. An enhanced excretion of D-glucaric acid occurs in patients under thiopental anaesthesia and in patients under treatment with phenobarbital-phenytoin, phenylbutazone or aminophenazone.

6. The bisphenylhydrazide of D-glucaric acid has been isolated from urine of a patient under treatment with phenobarbital and phenytoin and identified

chemically. The quantity isolated largely exceeds the quantity that may be expected on the basis of a normal D-glucaric acid excretion.

Chapter 5

7. It has been observed that 2 hours after barbital administration (i.p.) to the rat the L-ascorbic acid level in the liver is not enhanced, while 3 hours after administration it is enhanced. It has also been observed that in a 3-hr urine portion collected immediately after administration of the drug the level of L-ascorbic acid and D-glucaric acid is enhanced. The urinary excretions of L-ascorbic acid and D-glucaric acid in dogs are found to be increased within the first 2 hours after application (i.v.) of barbital and thiopental.

8. The effect of cumulative doses of barbital on the urinary excretions of L-ascorbic acid, D-glucaric acid and D-glucuronic acid has been studied in the rat. The response of L-ascorbic acid resembles that of D-glucaric acid. The excretion of D-glucuronic acid is enhanced during barbital treatment. The response of D-glucuronic acid is difficult to interpret because of the high pre-treatment level of this acid in the urine. The increase in the excretion of L-ascorbic acid has been observed even up to the 30th day after the last dose.

9. In individual untreated rats the amount of L-ascorbic acid excreted from day to day in the urine is rather constant. The amounts of L-ascorbic acid excreted by different rats of the same strain and sex, however, are found to vary considerably.

10. A single dose of barbital administered to rats results in an enhanced excretion of L-ascorbic acid and D-glucaric acid. A single dose or two doses of aminophenazone, nikethamide, borneol, phenylbutazone, thiopental, DDT, salicylic acid or tolbutamide results in an enhanced L-ascorbic acid excretion, but not in an enhanced D-glucaric acid excretion in the 24-hr period after drug application. A D-glucaric acid response becomes evident after treating rats during 3 to 4 days with aminophenazone, nikethamide, borneol or aminophenazone. An enhanced D-glucaric acid excretion is not observed in the 24-hr period after administration of high doses of aminophenazone or thiopental to guinea pigs.

11. Treatment of rats with D-glucuronolactone results in an enhanced D-glucaric acid excretion which is relatively strong as compared with a simultaneously occurring enhanced excretion of L-ascorbic acid. The results obtained with drugs and D-glucuronolactone in rats and guinea pigs may indicate that the D-glucaric acid pathway is not an intrinsic part of the glucuronic acid system.

12. Barbital, DDT or nikethamide, drugs stimulating the glucuronic acid system, do not enhance the glucuronidation in the 8-hr period following salicylamide administration to rats. The drug-induced stimulation of the glucuronic acid system as is obvious from the increased L-ascorbic acid excretion, and the enhanced glucuronidation as caused by drugs, appear not to be directly related.

Chapter 6

13. The UDP glucuronyltransferase activity, as measured within the first few days of drug treatment with the aid of body-foreign compounds, is not involved in the drug-induced stimulation of the glucuronic acid system.

14. An enhanced activity of hepatic UDPG dehydrogenase is observed during chronic treatment of rats with chloretone. After a short treatment (1–3 days) with barbital or chloretone no enhanced activity of this enzyme is observed. It is concluded that the drug-induced stimulation of the glucuronic acid system is not the result of an enhanced UDPG dehydrogenase activity.

15. It is observed that puromycin and actinomycin D do not prevent the enhanced excretion of L-ascorbic acid and D-glucaric acid after barbital administration. So the barbital-induced stimulation appears not to be related to *de novo* protein synthesis. It is argued that the same holds true as far as the stimulation of the glucuronic acid system by other drugs is concerned.

16. Many drugs cause stimulation of the glucuronic acid system as well as an enhanced drug-metabolizing capacity in the liver. The latter phenomenon is based on *de novo* protein synthesis. One of the starting points of this thesis was the notion that an enhanced D-glucaric acid excretion as a result of stimulation of the glucuronic acid system might be considered as a criterion for the occurrence of both an enhanced capacity to metabolize drugs and a stimulation of the glucuronic acid system in the liver. From the results summarized under 10 and 15 it is concluded that this notion is untenable.

Chapter 7

17. Rats were deprived of one of the following hormonal centres: adrenals, hypophysis, pancreas, gonades, glandulae thyreoideae and parathyreoideae. Treatment of these rats with barbital resulted in an enhanced excretion of D-glucaric acid and/or of L-ascorbic acid, indicating that the stimulation phenomenon is not effected via the hormonal centres mentioned.

18. An enhanced excretion of D-glucaric acid and L-ascorbic acid has not been observed in rats treated with the stress-agent celite (a diatomaceous earth). An enhanced activity of hepatic enzymes involved in D-glucuronic acid metabolism e.g. UDPG dehydrogenase and D-glucuronolactone dehydrogenase has not been observed either. Drug-induced stimulation of the glucuronic acid system appears not to be related to the stress situation initiated via the adrenals.

19. It is shown that during drug treatment male rats give a stronger response in the glucuronic acid system than female rats. The difference disappears upon castration and could be reversed by treating castrated female rats with testosterone and castrated male rats with estradiol.

20. On the basis of a study of literature a comparison is made between the drug-induced stimulation of the glucuronic acid system and the drug-induced stimulation of the pentose phosphate pathway. Both phenomena do not appear to be directly related. An outline of further experiments is given. These experiments are now being performed.

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STELLINGEN

I

Bij patiënten die behandeld worden met de geneesmiddelen barbital, aminofenazon of fenylbutazon treedt een verhoging in het gehalte aan D-suikerzuur van de urine op.

Aarts, E. M.: Biochem. Pharmacol. 14, 359 (1965)
Dit proefschrift

II

Aan de door farmaca veroorzaakte stimulering van het glucuronzuursysteem ligt geen verhoogde *de novo* synthese van enzymen ten grondslag.

Dit proefschrift

III

De door barbital veroorzaakte stimulering van het glucuronzuursysteem bij de rat houdt geen verband met de eveneens door barbital veroorzaakte vergroting van het vermogen van de rat om farmaca te glucuronideren.

Dit proefschrift

IV

De conclusie van Kato et al. dat de aan microsomen gebonden fractie van rattelever ribosomen toeneemt na behandeling van ratten met phenobarbital wordt niet gerechtvaardigd door hun experimenten.

Kato, R., Jondorf, W. R., Loeb, L. A., Ben, T. en Gelboin, H. V.: Mol. Pharmacol. **2**, 171 (1966)

V

Tegen de gevolgtrekking van Levy en Amsel dat benzoëzuur bij de mens de vorming van salicyluraat competitief remt zijn ernstige bezwaren aan te voeren.

Levy, G. en Amsel, L. P.: Biochem. Pharmacol. **15**, 1033 (1966)

VI

Door Ernst et al. is waargenomen dat bij de eend de ($Na^+ + K^+$)-ATPase-activiteit en de Mg^{++}-ATPase activiteit van homogenaten van de zoutklier afnemen na beëindiging van de belasting met zout. Deze waarnemingen kunnen niet dienen als argument voor het voorkomen van twee ATPasen in het betrokken weefsel.

Ernst, S. A., Goertemiller, C. C. en Ellis, R. A.: Biochim. Biophys. Acta **135**, 682 (1967)

VII

Voor de beantwoording van de vraag of lever reticulosomen betrokken zijn bij de vorming van de membranen van het endoplasmatische reticulum, is een vergelijkend onderzoek gewenst naar de aanwezigheid van ATPase en glucose-6-fosfatase in de reticulosomen en in de membranen van het granulaire en gladwandige endoplasmatische reticulum tijdens de verschillende ontwikkelingsstadia van de lever.

Pollak, J. K., Wand, K. en Shorey, C. D.: J. Mol. Biol. **16**, 564 (1966)
Pollak, J. K. en Ward, D. B.: Biochem. J. **103**, 730 (1967)
Dallner, G., Siekevitz, P. en Palade, G. E.: Biochim. Biophys. Res. Communs. **20**, 135 (1965)

VIII

De experimenten van Scholtan, die er op wijzen dat sulfonamide door bilirubine verdrongen wordt van de bindingsplaatsen aan eiwit zijn meer steekhoudend dan die van Josephson en Furst, waarbij geen invloed van bilirubine op de binding van sulfonamiden aan eiwit wordt vastgesteld.

Scholtan, W.: Arzneim.-Forsch. **11**, 707 (1961)
Josephson, B., en Furst, P.: Scand. J. Clin. Lab. Invest. **18**, 51 (1966)

IX

Het feit dat geen rekening is gehouden met de door Schach von Wittenau en Blackwood beschreven stereochemie van oxytetracycline ontneemt aan de discussie door Hussar et al. over de invloed van de 5-OH groep op de epimerisering van de tetracyclines elke zin.

Hussar, D. A., Niebergall, P. J., Sugita, E. T. en Doluisio, J. T.: J. Pharm. Pharmacol. **20**, 539 (1968)
Schach von Wittenau, M., Blackwood, R. K., Conover, L. H., Glauert, R. H. en Woodward, R. B.: J. Am. Chem. Soc. **87**, 134 (1965)
Schach von Wittenau, M. en Blackwood, R. K.: J. Org. Chem. **31**, 613 (1966)

X

Bij de door geautolyseerde plantaardige en dierlijke weefsels veroorzaakte celdifferentiatie in callusweefsel van Phaseolus vulgaris kan naast auxine ook cytokinine een rol spelen.

Sheldrake, A. R. en Northcote, D. H.: Planta **80**, 22 (1968)
Bergmann, L.: Planta **62**, 221 (1964)

XI

Aan de mogelijkheid een meer verantwoorde farmacotherapie in te stellen op geleide van bloedspiegels van geneesmiddelen wordt onvoldoende aandacht besteed.

XII

De opleiding tot apotheker dient dringend aangepast te worden aan de hedendaagse ontwikkeling op het gebied van de voorziening met en de toepassing van geneesmiddelen.